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(54) Title: SELECTIVE REGULATION OF ADENOVIRUS PRODUCTION

(57) Abstract

The present invention relates to adenovirus vectors and their use in DNA delivery systems. The vectors have been designed to maximize their capacity to carry foreign DNA and to minimize the potential of producing replication competent virus. The vectors contain one or more copies of a minimum packaging sequence to direct virus packaging. Optionally, the vectors contain one or more repressor binding sites so that virion production can be selectively inhibited. Specific repression systems include COUP-TF and *lac* repressor. A cellular complex, called P complex is also disclosed. This complex functions positively in viral packaging and virus production.

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SELECTIVE REGULATION OF ADENOVIRUS PRODUCTION

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FIELD OF THE INVENTION

This invention relates to the field of adenovirus vectors and the regulation of viral particle production. One area of application is in the field of DNA delivery systems.

BACKGROUND OF THE INVENTION

Adenovirus is a common human DNA virus that naturally infects the airway epithelia as well as other tissues in the body. Adenovirus ("Ad") is a particularly useful virus as a human DNA delivery system for a number of reasons. First, the genetic organization of the virus and functions of many virus-encoded gene products have been characterized. Second, the Ad genome is easily manipulated in the laboratory, and recombinant virus are readily grown to high titers in cultured cells. Third, Ad has a wide host cell range, and recombinant Ad vectors have been used to efficiently infect multiple cell types in culture and in animals. Adenoviruses have been shown to infect a variety of tissues in animal studies including liver, kidney, muscle, respiratory, endothelial and nervous system. Diseases that affect these and other tissues

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° therefore are amenable to adenovirus-mediated gene therapy. Fourth, Ad has the ability to efficiently infect non-dividing differentiated cells in the animal, a major target for DNA delivery applications. Finally, 5 adenovirus is a relatively benign human virus that is associated with mild disease, and importantly is not associated with the development of any human malignancy.

Adenovirus-based vectors offer several unique advantages, including tropism for both dividing and non- 10 dividing cells, minimal pathogenic potential, ability to replicate to high titer for preparation of vector stocks, and the potential to carry large inserts. The cloning capacity of an adenovirus vector is about 8-10 15 kb, resulting from the deletion of certain regions of the virus genome dispensable for virus growth, e.g., E3, deletions of regions whose function is restored in trans from a packaging cell line, e.g., E1, and its complementation by 293 cells (Graham, (1977)), and 20 deletion of E2b (Amalfitano, et al., (1998)) and E4 (Krougliak, et al. (1995); Brough, et al. (1996)) as well as the upper limit for optimal packaging which is about 105% of wild-type length.

25 Adenovirus DNA encapsidation occurs in a polar manner from left to right and relies on a cis-acting packaging domain located between approximately nt 200-380 (Daniell et al. (1976); Hammerskjöld et al. (1980); Hearing et al. (1987); Robinson et al. (1984); Tibbetts 30 (1977)). The location of the adenovirus type 5 (Ad5) packaging domain is schematically depicted in Fig. 1A. The Ad5 packaging domain consists of at least seven redundant, albeit not functionally equivalent, elements 35

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° termed A repeats I through VII (Graeble et al. (1990); Graeble et al. (1992)).

5 Little is known about the identity of trans-acting packaging components involved in Ad packaging, but several lines of evidence support their existence in limiting concentrations in the infected cell. Most notably, cotransfection of an excess of unlinked packaging domain sequences with wild-type adenovirus genomes dramatically decreases virus yield without a significant effect on DNA replication and late transcription (Graeble, et al. (1992)). This decrease in virus growth is thought to reflect the competition of limiting, trans-acting packaging components from viral genomes by the unlinked packaging domain fragments, resulting in their inability to be encapsidated.

10 A major goal in DNA delivery systems is to create a viral vector that lacks all viral coding sequences, and only contains DNA of interest for delivery purposes plus minimal viral DNA sequences required for growth and production of the virus. To grow such a virus, a helper virus is required, but selection against contamination of the virus stock with the helper virus (wild type virus) must be imposed. The only system described to date to selectively repress packaging of an adenovirus helper virus is the excision of the packaging domain using the CRE-LOX system. This system reduces packaging of the helper virus 100- or 30 500-fold (Parks, et al.(1996); Hardy, et al.(1997)).

35 In the field of adenovirus gene therapy, the risk of RCA (replication competent adenovirus) is a major concern of developers and the FDA. RCA is the generation of wild type, infectious adenovirus via the

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recombination between two different viruses within an infected cell. RCA arises primarily through homologous recombination between two viruses coinfecting in a cell between overlapping homologous DNA sequences, or between
5 virus DNA and viral DNA integrated into host chromosomes in certain complementing cell lines used to grow such a virus.

One object of the present invention is the identification of a specific control element which
10 mediates the function of the adenovirus packaging domain. This control element operates through binding of one or more trans-acting proteins.

Another object of the present invention relates to the identification of a minimum packaging
15 signal which can direct adenovirus packaging.

Yet another object of the present invention relates to adenovirus vector constructs having a selectively regulated packaging function.
20

Another object of the present invention relates to the use of adenovirus vectors with a regulated packaging function in a DNA delivery system.

It is another object of this invention to
25 provide selective repression of packaging of one virus, and not another, in the context of coinfection of two viruses into cells.

It is a further object of this invention to provide a novel means to specifically repress the
30 production of a helper virus while allowing the production of an adenovirus vector during the preparation of the virus.

Yet another object of the present invention
35 relates to repressor-mediated control of adenovirus

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particle production containing binding sites for such repressors and the use of vectors containing such binding sites for DNA delivery.

Finally, it is another object to reduce RCA in preparations of Ad virus by constructing such vectors and a helper virus with no overlap in the packaging sequences to eliminate homologous recombination.

SUMMARY OF THE INVENTION

The present invention relates to adenovirus vectors containing a minimum packaging signal for producing adenovirus virions. Of special importance is the presence of a CG dinucleotide located downstream of a TTTG sequence within each of the packaging elements. Spacing between the consensus segment 5'-TTTG-3' and the 5'-CG-3' segment located downstream is preferably between 1 and 12 nucleotides. Alternatively, it may be preferred to configure the consensus segments so that these elements appear on the same surface of the DNA helix. Most preferably, the adenovirus vector of the present invention may contain a packaging element consisting of 5'-TTTGN₈CG-3' which represents a minimal sequence necessary for adenovirus packaging. This sequence is preferably present in multiple copies. One type of minimal packaging sequence is an "A repeat", which contains a consensus sequence. Several A repeat sequences are shown in Table 1.

Another aspect of the present invention relates to novel vectors containing the minimum packaging sequences which can be selectively regulated. One such embodiment provides an adenovirus vector containing minimum packaging sequences and repressor

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° sites, such as COUP-TF or lac repressor sites. Such
vectors are selectively packaged in the absence of the
repressor. The repressor sites may flank the packaging
sequence, may be embedded into the packaging sequence or
5 may alternate the packaging sequence. Such vectors may
contain one type of repressor site or combinations of
different repressor sites.

The present invention also relates to a
cellular DNA binding protein, called COUP-TF, which
10 binds to adenovirus DNA packaging sequences. It has
been found that over-expression of COUP-TF in cells
infected with adenovirus specifically represses virus
production; in particular, virus packaging. COUP-TF
15 preferentially binds to certain packaging elements.
Thus, adenovirus vectors of the present invention may
contain one or more COUP-TF binding sites. Adenovirus
vectors of the invention may contain a combination of
COUP-TF binding sites and minimal packaging sequences.
20 These elements can be used to selectively regulate
packaging of such viruses.

The present invention relates to a method of
regulating adenovirus packaging comprising the steps of
25 obtaining an adenovirus vector containing a repressor
binding site, propagating this vector in the absence of
the repressor and repressing packaging of said vector in
the presence of COUP-TF. Such a method may be carried
out in one cell line. Alternatively, the propagating
30 step may be carried out in a first cell line and the
repressing step may be carried out in a second cell
line. In such a system, the repressor may be endogenous
to the cell line or exogenously provided at the DNA or
35 protein level.

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0 The present invention provides adenovirus
vectors that package the virus using one or more COUP-TF
binding sites or, for example, one or more A repeats.
Thus, the present invention provides a selective system
5 to control the packaging of an adenovirus vector.
Optionally, the system can be designed to allow
efficient packaging of one adenovirus vector while
inhibiting packaging of a different vector in the same
infected cell by using viruses with different packaging
10 sites and/or COUP-TF binding sites in conjunction with
COUP-TF over-expression.

 Yet another aspect of the present invention
provides a method of treating a patient through the
15 administration of a heterologous gene that is expressed
in the patient or a DNA fragment that is itself
therapeutically active in the patient. This gene or DNA
is delivered to the patient via an adenovirus vector
which is prepared for administration using a regulatable
20 adenovirus vector of the present invention.

 The present invention also relates to P-
complex, an activity involved in adenovirus packaging.
P-complex appears to contain TATA-binding protein
25 ("TBP") and TAF₁₇₂ and is useful in production or
packaging of viral particles. P-complex, interacts with
the minimum packaging signal of adenovirus.

BRIEF DESCRIPTION OF THE DRAWINGS

30 These and other objects, features and many of
the attendant advantages of the invention will be better
understood upon a reading of the following detailed
description when considered in connection with the
35 accompanying drawings herein.

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FIG. 1 depicts the adenovirus type 5 packaging domain. (A) A schematic representation of the left end of the adenovirus type 5 genome. Nucleotide positions are indicated by numbers. The inverted terminal repeat (ITR) is represented by a gray box. Viral packaging repeats are termed A repeats I to VII (arrows). The E1A transcriptional start site is indicated by an arrow, and enhancer elements I and II are designated as E1A enhancer. (B) The packaging repeat consensus motif. Shown is an alignment of A repeats I, II, V and VI. Nucleotides comprising the bipartite consensus motif for A repeats I, II, V and VI are boxed and enlarged. The consensus motif is shown at the bottom (5'-TTTGN₈CG-3'). (C) Alignment of A repeats V and VI in different adenovirus subgroups: Ad 5 (subgroup C), Ad 4 (subgroup E), Ad 12 (subgroup A), Ad 3 (subgroup B), Ad 9 (subgroup D). The positions of AV and AVI are shown by horizontal lines above the sequence. Nucleotides identical between all subgroups are indicated by vertical lines.

FIG. 2 depicts the functional hierarchy among different packaging repeats. A schematic representation of left-end sequences of wild-type adenovirus is shown at the top (as per Fig. 1A). A repeats AI, AII, AV and AVI are represented by boxes of distinct shading. The mutant viruses contain a deletion between nucleotides 194 and 814, and the insertion of 6 copies each of AVI (194/814:AVI6), AII (194/814:AII6) and AI (194/814:AI6), a dimerized copy of AV, AVI and AVII (194/811:AV-AVII2) or 12 copies of AVI (194/814:AVI12). Mutant virus yields in the single infections (Yield) are expressed as fold-reduction relative to that of the wild-type virus.

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0 The results from the coinfection experiments (Coinf.) are expressed as fold-reduction in packaged mutant DNA relative to packaged wild-type DNA. NV, virus was not viable. ND, packaged viral DNA was below the level of
5 quantitation.

FIG. 3 depicts a cellular complex (P complex) which interacts with adenovirus packaging elements. A gel mobility shift competition experiment is presented. Radio labelled probe (AV-VII dimer) 293 nuclear extract
10 and nonspecific competitor DNA (polydIdC) were incubated in the absence (lanes 1 and 24) or presence (lanes 2 to 23) of competitor oligonucleotides. P-complex DNA binding activity is indicated by an arrow. Increasing
15 amounts of specific competitor oligonucleotides are indicated, and represent a 40- and 200-fold molar excess of A repeats relative to the probe. The competitors are named according to the A repeats they represent. An LS was appended when the TTTG consensus motif in the
20 oligonucleotide was mutated. A CG was appended when the CG consensus dinucleotide was mutated.

FIG. 4 depicts P-complex and adenovirus DNA packaging. The left terminus of the adenovirus genome
25 is schematically represented with ITR and packaging domain denoted by boxes. Trans-acting components binding ITR and packaging sequences are identical in the model on the left, whereas different factors interact with the respective sequences in the model on the right
30 as indicated by circles.

FIG. 5 depicts the scheme used for P-complex purification.

FIG. 6 depicts the binding of COUP-TFI to
35 minimal packaging domains. Gel mobility shift assays

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0 were performed using COUP-TFI synthesized by *in vitro*
translation. A hexamer of A repeat VI (lanes 1-9) and a
hexamer of A repeat I (lanes 10-18) were used as
radiolabelled probes. Unprogrammed reticulocyte lysate
5 (Unprog) or increasing amounts of COUP-TFI-programmed
lysate (COUP) was used in binding reactions. The
addition of preimmune serum (P) or anti-COUP antiserum
(α -COUP) is indicated above the lanes.

10 FIG. 7 depicts multimerized oligonucleotides
corresponding to A repeats AI and AVI used to construct
recombinant viruses. A dimeric oligonucleotide sequence
is shown to simplify the schematic diagram. The
potential COUP-TF binding sites in the oligonucleotides
15 are indicated by arrows. Perfect or 4-out-of-5
nucleotide matches to the COUP-TF consensus sequence are
shown as closed arrowheads; 3-out-of-5 nucleotide
matches to the COUP-TF consensus site are shown as open
arrowheads. Perfect, or nearly-perfect, COUP-TF binding
20 sites with a 1 base spacing are found in multiple
locations in the AVI oligonucleotide repeat, but not in
the AI oligonucleotide repeat.

25 FIG. 8 depicts a scheme for growth of a
"gutted" adenovirus gene therapy vector and the specific
repression of packaging of a helper virus needed to grow
the "gutted" virus. The "gutted" adenovirus lacks viral
coding regions and contains the inverted terminal
30 repeats (ITRs) required for DNA replication and a
hexamer of A repeat I (for example) to direct viral DNA
packaging. The remainder of the recombinant adenovirus
vector is available for the insertion of large DNA
segments (28 to 36 kbp). The helper virus carries all
35 of the wild type adenovirus genome and the packaging

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° domain is replaced with multimerized copies (12) of A repeat VI. The helper virus is grown without COUP-TF1 overexpression to allow for the high level production of the helper virus. For the production of the "guttet"
5 adenovirus, cells that overexpress COUP-TF1 are coinfectd with the "guttet" adenovirus and the helper virus. The helper virus allows for the production of Ad early and late gene products for complementation in trans of the "guttet" adenovirus. However, the
10 packaging of the DNA genome of the helper virus is specifically repressed by COUP-TF1 overexpression, while packaging of the genome of the "guttet" adenovirus is not repressed since its packaging elements do not bind COUP-TF1.
15

Fig. 9 depicts the specific repression of packaging of a "designer" adenovirus vector by expression of COUP-TF. (A). The growth of adenovirus USFO was measured without or with expression of COUP-TF. 293 cells were cotransfected with USFO DNA plus increasing concentrations of empty expression vector (CMX) or an expression vector for high level production of COUP-TF (CMX-COUP-TF). Virus yield (log virus yield)
20 was measured by plaque assay on 293 cells. COUP-TF expression had a minimal effect of production of the USFO virus. (B). The growth of adenovirus USFO+AVI¹² was measured, as described in (A). COUP-TF expression specifically repressed production of the "designer" virus USFO+AVI¹². The maximum level of repression of packaging of USFO+AVI¹² by COUP-TF expression was 400-fold. (C). Western blot analysis of adenovirus late protein expression without or with COUP-TF expression.
30 293 cells were cotransfected with USFO DNA without or
35

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with expression of COUP-TF. Adenovirus late protein fiber and penton were quantified by Western blot using specific antibodies. The results show COUP-TF expression has a minimal effect on adenovirus late gene expression.

FIG. 10 depicts synthetic oligonucleotides that contain different adenovirus packaging repeats designed with specific repressor binding sites that either overlap the packaging A repeats or are placed between packaging A repeats. (A) The sequence of the wild type AV-AVII oligonucleotide. A dimeric copy of this oligonucleotide efficiently directed packaging in a recombinant virus (Fig. 2). A repeats V, VI and VII are indicated and the consensus packaging repeats are encircled. (B) The AV-AVII oligonucleotide is modified (underlined nucleotides) to create a high affinity binding site for the adenovirus-induced E2F-E4-6/7 protein complex overlapping A repeats V and VI (binding site indicated by inverted arrows). (C) The AV-AVII oligonucleotide is modified (underlined nucleotides) to create a high affinity binding site for the *E. coli* lac repressor overlapping and adjacent to A repeat V (binding site indicated by inverted arrows).

Fig. 11 (A) Western blot showing lac repressor expression in 293 cells and (B) gel mobility shift assay showing lac repressor protein expressed in 293 cells binds to the AV-AVII + lac site shown in Fig. 10C.

Fig. 12 depicts the specific repression of packaging of a "designer" adenovirus vector by expression of lac repressor. The growth of adenovirus AV-VII+lac was measured without or with expression of lac repressor. 293 cells were cotransfected with AV-

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° VII+lac DNA plus increasing concentrations of empty
expression vector (CMX) or an expression vector for high
level production of lac repressor (CMX+lac repressor).
Virus yield (log virus yield) was measured by plaque
5 assay on 293 cells. Lac repressor expression
specifically repressed production of the "designer"
virus AV-VII+lac. The maximum level of repression of
packaging of AV-VII+lac by lac repressor expression was
20-fold.

DETAILED DESCRIPTION OF THE INVENTION

10 The present invention relates to regulation of
adenovirus packaging. Both *cis*- and *trans*-acting
elements are described. These elements control
15 adenovirus packaging, and as such, their selective use
in adenovirus vectors for DNA delivery can reduce the
danger of producing RCA in viral preparations and in
patients.

20 The present invention is directed to
regulatable adenovirus vectors. These new vectors have
specific packaging sequences and are regulated so that
production of viral particles is controlled. The vector
25 design also increases the safety of recombinant
adenovirus vectors for use as DNA transfer vehicles by
reducing the potential for RCA.

30 The adenovirus vectors of the present
invention may be derived from any known adenovirus
serotype. The A repeats used as minimum packaging
sequences may also be derived from any adenovirus
serotype. Several example A repeats and their
similarity between serotypes are illustrated in Figure
35 1C.

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0 One aspect of the invention identifies that a
COUP-TF binding site acts as an active site for
repression of adenovirus packaging. Conversely, another
aspect of the invention identifies a complex, termed P-
5 complex which is involved in packaging. Packaging is a
critical function of the adenovirus for production of
viral particles. One important use for a regulated
adenovirus vector is in the field of DNA delivery for
therapeutic applications which uses a viral vector to
10 deliver genes or DNAs of interest to a patient in need
of such treatment.

"DNA delivery system" as this term is used
herein refers to a system of delivering a DNA to a
15 patient. Such a DNA may contain a gene encoding a
protein whose expression in the patient may provide a
therapeutic benefit. Such proteins may, for example,
act as a treatment for a disease or condition, or may
stimulate an immune response, such as a vaccine. Gene
20 therapy is one such DNA delivery system. Alternatively,
the DNA of interest may not encode a protein yet may
provide a benefit to the patient. For example, a DNA
may act as a antiviral agent or may transcribe into an
RNA which may act as an antisense therapeutic or
25 antiviral agent.

The present invention also relates to the
identification of a minimum adenovirus packaging signal.
A minimal packaging sequence of 5'-TTTGN₈CG-3' has been
30 identified. Although eight nucleotides are preferred to
separate the left portion of the packaging consensus
element (i.e., 5'-TTTG-3') from the right portion (i.e.,
5'-CG-3'), this spacing may vary 1 to 12 nucleotides.
35 Alternatively, it may be preferred to configure the

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° consensus segments so that the segments appear on the same surface of the DNA helix. The packaging element may be inserted into the left or right end of the adenovirus vector, preferably within 600 nucleotides from either end. More preferably, this minimal sequence is present at the left end of the genome and is present in multiple copies. Another consensus sequence comprises 5'-ATTGNG₈CG-3' and provides a strong packaging signal in adenovirus vectors. Two copies of this minimal packaging sequence are sufficient to ensure packaging. More than two copies enhance virus packaging. However, any number of this sequence can be inserted into the virus to ensure particle production. "Multimerized" as this term is used in the instant application refers to multiple copies of an element (i.e. packaging or repressing). These elements may be present in single units or in multimers, which preferably means 2-36 repeats and more preferably 2-12 units or elements. One form of the minimal packaging element is an "A repeat", which is derived from adenovirus. Representative A repeats are set forth below in Table 1:

TABLE 1

25	AI:	5'-TTTGGGCGTAACCG-3'
	AII:	5'-TTTGGCCATTTTCG-3'
	AIII:	5'-TCTGAATAATTTTG-3'
	AIV:	5'-TTTGTGTTACTCAT-3'
30	AV:	5'-TTTGTCTAGGGCCG-3'
	AVI:	5'-TTTGACCGTTTACG-3'
	AVII:	5'-TTTACGTGGAGACT-3'

Unique adenovirus vectors that contain minimal packaging domains have been developed consisting of

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multimerized oligonucleotide sequences in place of the normal packaging domain. Additionally, these new adenovirus vectors may contain deletions of viral DNA sequences from the left end of the genome which allow for augmented insertion of foreign DNA sequences in the context of DNA delivery vectors. Up to 400 nucleotides can be deleted from the left end of the genome and be replaced with the minimum packaging sequences defined herein to produce a vector with an increased capacity to carry foreign DNA. Further, the use of different packaging oligonucleotide repeats in different individual viral vectors allows for the selective repression of packaging of one adenovirus vector, but not another adenovirus vector, in cells coinfecting with both viruses. The latter scenario is important in the design of a vector capable of selective packaging for use in DNA delivery systems, and the repression of packaging of a helper virus needed to grow the adenovirus vector.

The vectors of the present invention are useful in DNA delivery systems to help curb the production of replication competent adenovirus (RCA), a virus that is dangerous and potentially toxic to a patient receiving it during patient administration. This is due to the fact that two distinct viruses can be made with entirely distinct, and non-overlapping packaging domains. For example, a virus (eg. gutted gene therapy virus 1) may contain a hexamer of A repeat I in direct orientation, while a helper virus (virus #2) may contain a dimer of A repeats V, VI and VII or a multimer of AVI in an inverted orientation. Thus, both viruses carry functional packaging domains, but overlap

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° homologous recombination is greatly minimized since different packaging sequences and DNA orientations are used. A target for homologous recombination does not exist in the packaging domain. In such coinfection
5 conditions, the use of different packaging domains in the two viruses greatly minimizes the possibility of recombination between the two viruses to generate RCA.

In one embodiment of the present invention, one or two copies of a DNA segment containing packaging
10 A repeats V, VI and VII direct packaging. A single copy of the segment functions for packaging. This type of packaging sequence contains a series of different repeats and is referred to as a natural packaging
15 domain. The second type of packaging sequence contains a single type of A repeat which when multimerized functions efficiently for packaging. This segment is referred to as a synthetic packaging element. Vectors of the present invention may contain a combination of
20 natural and synthetic packaging elements.

The present invention approach to DNA delivery vector design preferably uses a "gutted" adenovirus vector whereby most or all of the viral genes are
25 removed. There are two advantages with "gutted" vector approach. First, little or no viral proteins are produced following infection that normally elicit an immune response. Second, such a virus is capable of carrying very large gene inserts for gene therapy
30 applications. For example, the dystrophin gene for treatment of muscular dystrophy is 14,000 bp in length necessitating a vector with very large insert capacity. Also, the Factor VIII gene for treatment of hemophilia A
35 is greater than 7000 bp. Additionally, it may be

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° preferable to use tissue-specific regulatory sequences to produce tissue-specific expression of a gene. This requires increasing the insert capacity in a vector, because many tissue-specific promoters contain several
5 thousand base pairs.

Many genes and/or DNA segments may be carried by adenoviral vectors. Examples of such genes include; interleukin-2 (Haddada, et al. (1993)) p53 (Harris, et al. (1996)); α 1-antitrypsin (Jaffe, et al. (1992)), cystis
10 fibrosis transmembrane conductance regulator (CFTR) (Rosenfeld et al., (1992)), and clotting factor VIII (Connelly, et al. (1995)).

The recombinant adenovirus of the present invention is preferably a "gutted vector" and contains adenovirus sequences at the left and right termini required for DNA replication and two or more copies of the minimal packaging sequence to direct viable DNA
15 packaging. The remainder of the recombinant adenovirus vector is available for insertion of large DNA segments (up to 36,000 base pairs). A helper adenovirus is needed to grow such a "gutted" vector in order to produce all of the viral proteins that are missing in
20 the "gutted vector".

In DNA delivery systems, there are circumstances in which it is desirable to prevent production of a viral particle. In particular, helper virus, a virus necessary for replication of the viral
30 construct, is highly undesirable in the preparation for patient administration. According to one embodiment of the present invention, a helper virus is designed to contain a COUP-TF binding site and is first allowed to
35 grow productively in the absence of COUP-TF, then is

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° blocked from being packaged by the presence of COUP-TF. In this embodiment, the viral growth is carried out in a cell line which does not express COUP-TF and the packaging is blocked by the addition of COUP-TF protein.

5 In a second embodiment, the viral growth is carried out in a cell line lacking COUP-TF (Qiu, et al. (1997)) and the packaging repression step is accomplished by transfer of the virus into cells expressing COUP-TF. In this way, helper virus can be used to propagate the

10 adenovirus vector yet not be present in the final viral preparation.

Another important aspect of the present invention relates to gene therapy vectors that use

15 adenovirus minimal packaging sequence, 5'- TTTGN₈CG -3'. (See Provisional patent application no. 60/081,867, incorporated herein by reference).

One preferred adenovirus vector design of the present invention utilizes a packaging/repressor system.

20 In this embodiment, adenovirus vectors are constructed with alternating oligonucleotides containing the minimal packaging sequence and binding sites for a repressor. For example, a lac repressor site can be inserted

25 between packaging sequences. The lac repressor is a high affinity binding repressor not found in eukaryotic cells. Another example of such a system embeds one or more repressor sites within a packaging domain. Yet another example of a packaging/repressor system flanks a

30 packaging domain with surrounding repressor binding sites. This system may have one or a series of repressor binding sites to the left of a minimal packaging domain and another set of repressor binding

35 sites to the right of a packaging domain. Thus, a virus

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° which contains minimal packaging sequence and repressor binding sites such as, for example, lac repressor sites, can be grown in cells not expressing the repressor, and then packaging can be selectively repressed in cells
5 expressing high levels of the repressor.

The present invention also provides vectors containing a packaging sequence in combination with the COUP-TF repressor binding sites whose packaging capability can be selectively controlled. For example,
10 such vectors may have a packaging sequence containing a dimer of A repeats V, VI. These packaging domains may also contain a COUP-TF repressor site as well as signals sufficient to allow efficient packaging. Such vectors
15 allow packaging in the absence of COUP-TF repressor, but inhibit packaging in the presence of COUP-TF.

For the production of the recombinant adenovirus of the present invention, cells that overexpress COUP-TF1 can be infected with the
20 therapeutic adenovirus vector containing one type of packaging element (for example, multiple copies of A repeat I) and the helper Ad containing a different type of packaging element (for example, multimerized copies
25 of A repeat VI). The packaging of the helper virus will be specifically suppressed by COUP-TF1 overexpression, while packaging of the genome of the adenovirus gene therapy vector will not be repressed. A conditional system for repression of packaging is designed into the
30 vector so that a helper virus can be grown to high levels under non-repression conditions, and then specific repression of the helper virus packaging accomplished when used to complement growth of the
35 therapeutic virus vector.

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In another vector embodiment of the present invention, *lac* repressor binding sites are embedded within a minimal packaging domain. For example, a packaging domain may be engineered to contain a *lac* repressor binding site embedded within the A repeat V, VI and VII packaging domain. The virus can then grow in the absence of *lac* repressor expression while repression of packaging (e.g. a helper virus) is observed with high level *lac* repressor expression. The virus can then grow in the absence of *lac* repressor expression while packaging is repressed when *lac* is expressed.

In yet another vector embodiment of the present invention, E2F transcription factor binding sites are embedded within a minimal packaging domain. The idea is the same as directly above, i.e. a high affinity binding site for a DNA binding protein is embedded within a minimal packaging domain with the ability to selectively "activate" the repressor. In this embodiment, the cellular transcription factor (E2F) and an adenovirus protein (E4-6/7) which induces the cooperative and stable binding of E2F to an inverted binding site provide the packaging/repressor system of this vector. A high affinity E2F inverted binding site is inserted within a minimal packaging domain containing, for example, A repeats V, VI and VII. In the absence of 6/7 protein expression (this mutant virus is completely viable), E2F binding to the packaging region is weak and thus repression is weak. In the presence of the E4-6/7 protein, E2F binding is stable and with high affinity. Thus, binding of the bona-fide packaging factor is repressed and packaging of the virus is blocked.

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0 Alternatively, binding sites for other
repressors, such as, for example, lambda repressor or
Tet repressor, can be employed in the design of
adenovirus vectors of the present invention. Other
5 potential repressor sites can be employed and will be
readily known to the skilled artisan.

 Multimers of different A repeats are able to
direct packaging of viral DNA but at different
efficiencies (Schmid and Hearing, 1998). Any of the A
10 repeats may serve as a minimal packaging sequence.
Preferably these A repeats are used as multimers in a
packaging element. A dimer of A repeats V-VII and a
hexamer of A repeat I, most preferably as a multimer,
15 serve as the most efficient packaging domains *in vivo*.
A hexamer of A repeat II can also be used in the present
invention, having a moderate activity. A hexamer of A
repeat VI is also a packaging element, albeit a weak
element. A repeat VI, when utilized as a multimer,
20 preferably a 12-mer, efficiently directs packaging.

 One embodiment of the present invention
relates to vector constructs containing multimers of the
A repeat VI packaging signal which is a high affinity
25 binding site for COUP-TF binding. Such a vector
construction can be regulated through selective
expression of COUP-TF.

 In light of the fact that COUP-TF binds to
adenovirus packaging sequences, the effect of
30 overexpression of COUP-TF on adenovirus infection was
tested. Overexpression of COUP-TF resulted in a 10,000-
fold decrease in the production of infectious
adenovirus. This effect was, at least in part, due to
35 repression of the adenovirus major late promoter ("MLP")

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° which directs the synthesis of adenoviral late mRNAs and thus viral late proteins. COUP-TF binds to a specific DNA sequence in the MLP that overlaps the binding site for the activating transcription factor called USF
5 (Sawadogo and Roeder, 1985). COUP-TF is a known repressor of eukaryotic transcription promoter activity (Cooner et al., 1992; Tsai and Tsai, 1997).

In another embodiment of the invention, P-complex was found to interact with cellular complexes in
10 the viral packaging machinery (Schmid and Hearing, 1998). A direct correlation is seen between the binding affinity of P-complex for different A repeats *in vitro* and the ability of the respective fragments to support
15 DNA packaging *in vivo*. The TTTG, but not the CG, packaging consensus half site is critical for P-complex interaction. In addition the P-complex binds to core replication sequences in the inverted terminal repeat (ITR). The cellular P-complex activity, by virtue of its
20 ability to interact with both packaging and core replication sequences, constitutes a *trans*-acting link between viral DNA replication and encapsidation. The binding of a cellular transcription factor, COUP-TF, to
25 minimal segments of the viral packaging domain was also detected. Its binding affinity does not correlate with viral DNA packaging *in vivo*, but rather repression thereof.

Cellular P-complex is a bona-fide adenovirus
30 packaging component. This complex appears to contain a TATA binding protein (TBP) and a second protein called TAF172 (Timmers et al. 1992, Taggart et al. 1992). P-complex binding is inhibited by ATP and magnesium.
35 Complex formation is observed on all minimal packaging

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domains that exhibit functional activity *in vivo*. The affinity of the P complex for the different multimeric A repeats *in vitro* correlates well with the ability of the respective *cis*-acting sequences to support viral DNA packaging *in vivo*. Specifically, AI and AV-VII constitute strong P complex binding sites and they confer maximal packaging activity *in vivo*. The most preferred P-complex binding sites comprise a hexamer of AI and a dimer of AV, AVI and AVII. On the other hand, AVI is noted as a weak binding site for P complex *in vitro*, and it serves as a particularly weak packaging domain *in vivo*. As discussed above, the Ad packaging consensus motif is a bipartite sequence with a conserved AT-rich and a GC-rich half site (5'TTTGN₆CG-3') (Schmid, et al. (1997)).

The identification of the DNA binding activity of P complex as containing TBP-TAF172 has important implications for the development of "designer" adenovirus vectors for repression of packaging. For example, using the viruses depicted in Fig. 8, a gutted gene therapy vector may be generated that binds P complex/TBP-TAF172 poorly using mutations in the AT-rich binding site that reduce TBP binding to DNA in the helper virus packaging sequences. Additionally, so-called "altered-specificity" TBP mutants may be used in the present invention (Strubin and Struhl, 1992). Such mutations produce TBP protein having altered specificity for binding to certain DNAs. That is, the altered-specificity TBP mutant binds to a TATA box sequence with a nucleotide change (TATA to TGTA), whereas the normal wild type TBP in the cell is unable to bind such a TGTA site efficiently. Thus, adenovirus vectors with altered

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° specificity P complex/TBP-TAF172 binding sites may be constructed to conditionally repress packaging of a helper virus. The helper virus contains the altered specificity TGTA binding site in place of the AT-rich part of the A repeat; the virus can be successfully propagated when altered-specificity TBP is provided in cells, and packaging of the helper virus repressed when grown in cells lacking the altered-specificity TBP. Other manipulations of the P complex/TBP-TAF172 binding site and/or manipulations of the DNA binding proteins can be made by the skilled artisan toward the same goal.

Our working model, shown in Figure 4, is based on the data from protein binding studies presented in the Examples. A coordinate interaction of packaging factors with viral A repeats is shown. Three copies of A repeats are preferred for efficient DNA encapsidation (Graeble et al. (1990); Graeble et al. (1992)), which likely reflects the need for the presence of multiple protein binding sites. Either the same or a different trans-acting component may bind the left-end 13 nt of the adenovirus genome. Physical association between the components bound to ITR and packaging sequences results in the formation of a nucleoprotein complex within the viral left end, marking the respective molecule as a bona-fide packaging substrate. This complex corresponds to the P-complex detected in our gel mobility shift assays since it exhibits binding specificity for both packaging and ITR sequences. The AT-rich packaging consensus half site is implicated in the initial recognition of A- repeats by packaging factors. Perhaps the CG-rich half site and proteins bound to it are involved in secondary events like capsid recognition or

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0 insertion of the viral DNA into the capsid. It is
noteworthy that the 8 bp spacing, or one helical turn of
the DNA, which separates the AT-rich and the CG-rich
consensus half site is important for DNA encapsidation
5 in vivo. This may reflect the need for a physical
interaction between components of the P-complex and
CG-bound unidentified components, to allow for the
timing and/or coordination of successive steps in
adenovirus DNA packaging.

10 While the invention is described above in
relation to certain specific embodiments, it will be
understood that many variations are possible, and the
alternative materials and reagents can be used without
15 departing from the invention. In some cases such
variations and substitutions may require some
experimentation, but will only involve routine testing.

The foregoing description of the specific
embodiments will fully reveal the general nature of the
20 invention and others can, by applying current knowledge,
readily modify and/or adapt for various applications or
such specific embodiments without departing from the
generic concept, and therefore such adaptations and
25 modifications are intended to be comprehended within the
meaning and range of equivalents of the disclosed
embodiments.

30 All articles, patents or other references
cited or referred to herein are hereby incorporated
herein in toto by reference.

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EXAMPLESMATERIALS AND METHODS

Virus constructions. Ad5 dl309, the parent for all the viruses described in this report, is a phenotypically wild type virus that contains a unique XbaI cleavage site at 3.8 map units (Jones, et al. (1979)). Plasmid pElA-194/814 contains the left end Ad5 XbaI fragment (nt 1-1339) with a deletion between nt 194 and 814 and a unique XhoI restriction site at the junction of the deletion. A head-to-tail hexamer of an oligonucleotide containing A repeat VI (5'-TCGACCGCGGGGACTTTGACC-3': 5'-TCGAGGTCAAAGTCCCCGCGG-3') was cloned into the 194/814 deletion. Similarly, head-to-tail hexamers of oligonucleotides containing A repeat I (5'-TCGAGTTGTAGTAAATTTGGG-3': 5'-TCGACCCAAATTTACTACAAC-3') or A repeat II (5'-TCGACCGAGTAAGATTTGGCC-3': 5'-TCGAGGCCAAATCTTACTCGG-3') were cloned into the pElA-194/814 background. pBR-194/814 and pBR-53/814 have sequences between nt 194 and 814 and nt 53 and 814 deleted. A monomer and dimer of viral sequences is located between nt 334 and 385 which contain AV, AVI, and AVII was cloned into the 194/814 deletion. A dimer of the nt 334 to 385 fragment as well as 12 head-to-tail copies of an oligonucleotide containing AVI (5'-TCGACCGCGGGGACTTTGACC-3': 5'-TCGAGGTCAAAGTCCCCGCGG-3') were cloned into the 53/814 deletion in either orientation. All mutations were verified by nucleotide sequence analysis.

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0 The recombinant plasmids were subsequently
rebuilt into intact viruses by the method of Stow
(1981). Viruses were amplified and titered on 293 cells.
Mutant viruses were screened by restriction analysis of
5 viral DNA obtained from infected 293 cells by the Hirt
procedure (Hirt (1967)), and all insertions were
verified by nucleotide sequence analysis of viral DNA
using PCR-based sequencing.

10 **Cultured cells and infections.** Virus stocks
were generated by three freeze-thaw cycles of infected
cell lysates and titered by plaque assays on 293 cells.
Virus infections were performed at a multiplicity of
infection (MOI) of 3 PFU per cell for 1 h at 37°C. Cells
15 were then washed twice with tris-buffered saline
solution and overlaid with fresh medium.

**Determination of virus yield and packaging
efficiency.** Both assays were performed as described
20 previously (Schmid, et al. (1997)). For the
determination of virus yield in a single infection,
infected cell lysates were prepared 48 h post-infection
and the amount of infectious virus was determined by
plaque assays on 293 cells. Packaging efficiency of the
25 mutant viruses was tested in a coinfection of 293 cells
with both mutant and wild-type dl309 virus. Forty-eight
hours post-infection, one half of the cells was used to
isolate total nuclear DNA, the other half was used for
the preparation of viral DNA from purified virions. Both
30 DNA preparations were digested with XbaI to distinguish
between mutant and wild-type DNA and quantitated by
Southern blot hybridization using pElA-WT, ³²P-labeled by
the random primer method (Feinberg, et al. (1983)), as a
35 probe. The relative intensities of the bands in

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0 autoradiograms were determined by densitometric
scanning. Quantitation of the data was performed by
using the public domain NIH Image program (written by
Wayne Rasband at the National Institutes of Health and
5 available from the Internet by anonymous ftp from
zippy.nimh.nih.gov or on floppy disk from NTIS, 5285 Port
Royal Rd., Springfield, VA 22161, part no. PB93-504868).
The data presented for virus yield in the single
10 infections and the data for packaging efficiency based
on coinfection experiments represent the averages of
three to five independent experiments.

The data presented for virus yield in the
single infections and the data for packaging efficiency
15 based on coinfection experiments represent the averages
of at least three independent experiments.

**Extract preparation and gel mobility shift
assays.** Nuclear extracts were prepared by the method of
Dignam and Roeder (1983), and dialyzed overnight against
20 mM N-2hydroxyethylpiperazine-N'-2-ethanesulfonic acid
20 (HEPES; pH 7.5), 100 mM NaCl, 10% glycerol, 5 mM MgCl₂,
0.2 mM EDTA, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl
fluoride (DB-100). The dialysate was cleared by
25 centrifugation at 25,000 x g for 20 minutes. Two to five
grams of nuclear extract was incubated with 0.5µg of
polydeoxyinosinic-deoxycytidylic acid (poly dIdC) and
20,000 cpm of ³²P-labeled probe DNA (2.5 to 5 fmol of
DNA) per *in vitro* binding reaction. The binding reaction
30 was carried out in a total volume of 20 µg for 1-2 hr at
room temperature in 40 mM HEPES pH 7.5, 70 mM NaCl, 0.1
mM EDTA, 0.5 mM DTT, 0.5 mM
phenylmethylsulfonylfluoride, 10 µg/ml BSA and 4%
35 Ficoll. The complexes were resolved electrophoretically

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at 10 V/cm on a 3.5% 30:1 (acrylamide: bisacrylamide) polyacrylamide gel in 0.5x TBE (25 mM Tris pH 8.3, 25 mM boric acid, 0.5 mM EDTA) at 4°C. For gel mobility shift assays performed with *in vitro* translated COUP-TFI protein, 0.25-1.5 µl of rabbit reticulocyte extract programmed with *in vitro* synthesized RNA transcript encoding COUP-TFI was assayed using the binding conditions described above. *In vitro* transcription and translation was performed as recommended by the manufacturer (Promega). For gel mobility supershift experiments, 0.5 µl of a rabbit polyclonal anti-COUP antiserum (a gift from Dr. Alonzo D. Garcia) was added after a one hour binding reaction, and incubation was then continued for an additional 30 minutes.

Plasmids, probes and competitor fragments.

Head-to-tail hexamers of A repeats I and VI, individually, and a dimer of A repeats V-VII were cloned into pUC9. The sequence of a monomer of A repeat I is: 5'-TCGAGTTGTAGTAAATTTGGG-3': 5'-TCGACCCAAATTTACTACAAC-3', a monomer of A repeat VI is: 5'-TCGACCGCGGGGACTTTGACC-3': 5'-TCGAGGTCAAAGTCCCCGCGG-3'. A monomer of AV-VII is: 5'-TCGACCGCGTAATATTTGTCTAGGGCCGCGGGACTTTGACCGTTTACGTGGAGAC T CC-3':5' TCGAGGAGTCTCCACGTAAACGGTCAAAGTCCCCGCGGCCCTAGACAAATATTACG CGG-3'. The fragments were liberated from the vector by digestion with EcoRI and HindIII, gel purified and ³²P-end-labeled with Klenow DNA polymerase and (α-³²P)dATP. For the preparation of ITR 1-13 probe, a monomeric oligonucleotide representing the left end 13

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° nt flanked by Xho/Sal linkers
(5'-TCGACATCATCAATAATC-3':5'TCGAGATTATTGATGATG was
end-labeled in the same way using (α -³²P)dCTP.

For the preparation of competitor fragments
5 containing packaging repeats, monomeric oligonucleotides
were multimerized using T4 DNA ligase. Selection for
head-to-tail multimers was achieved by subsequent
digestion using SalI and XhoI, followed by
phenol/chloroform extraction and ethanol precipitation.
10 In addition to multimers prepared from the
oligonucleotides representing packaging elements I, VI
and V-VII described above, A repeat II (5'-
TCGACCGAGTAAGATTTGGCC-3':5'-TCGAGGCCAAATCTTACTCGG-3')
15 and A repeat V (5'-TCGACCGCGTAATATTTGTCC-3':
5'-TCGAGGACAAATATTACGCGG-3') were used as multimeric
competitors. Packaging repeat competitor fragments
designated LS have the underlined nucleotides shown
above in AI, AII, AV, AVI, AV-VI mutated into the
20 sequence 5'GTGCAG-3' (only the upper strand is
indicated). The italicized CG dinucleotide in the AV
competitor was replaced by an AT in the competitor
fragment designated CG. The competitor oligonucleotide
25 representing ITR sequences 1-13 was used in monomeric
form and was identical to the one used for probe
preparation. The monomeric ITR 10-22 competitor
oligonucleotide contains sequences between Ad nt 10-22
flanked by XhoI/SalI linkers. Quantitation of
30 oligonucleotide competitors was performed
spectrophotometrically. The amount of specific
competitor DNA added per binding reaction is indicated
in the text as -fold molar excess of binding sites
35 present in the competitor relative to binding sites

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present in the probe. This definition, however, is based on the assumption that one binding site (located between nt 1-13) is present in monomeric ITR fragments and that six binding sites are present in hexameric packaging repeat fragments.

Western blot analysis. Proteins separated by SDS-polyacrylamide gel electrophoresis were transferred to nitrocellulose, and probed with different antibodies (rabbit polyclonal anti-COUP, anti-fiber and anti-penton antisera, monoclonal antibody M45). Proteins were visualized using a secondary horseradish peroxidase-conjugated antibody and chemiluminescence as recommended by the manufacturer (Amersham).

EXAMPLE 1

Minimal adenovirus packaging domains.

Adenovirus packaging elements are functionally redundant, but in spite of this redundancy, different elements are not functionally equivalent with respect to each other. Elements I, II, V and VI constitute the most functionally dominant A repeats (Graeble et al. (1990); Graeble et al. (1992); Schmidt et al. (1997)). The selection of revertant adenoviruses from a packaging deficient parent virus has been defined A repeat VI as an independent cis-acting unit (Schmid, et al. (1997)). A hexamer of A repeat VI in place of the packaging domain yields a viable virus, although the mutant is reduced >100-fold in growth compared to wild-type. Such a mutant is under strong evolutionary pressure for the amplification of packaging elements since revertants with significantly improved growth were found to evolve by amplification of preexisting copies of A repeat VI.

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° In contrast, a fragment containing A repeats V-VII functions efficiently to direct packaging and these A repeats did not amplify upon virus propagation (Fig. 2; Schmid, et al. (1997)). Sequences flanking the
5 packaging domain are identical in both of these mutant viruses (a deletion of sequences between nt 194 and 814). This raises the question of whether there is a hierarchy of importance among the four most- dominant A repeats with A repeat VI as a functionally less dominant
10 element, or alternatively, whether a combination of different elements supports packaging better than only one type of A repeat.

To begin to address the first possibility,
15 viral mutants were constructed that contain multimers of individual A repeats inserted into a 194/814 deletion background (Fig 2) (Schmid and Hearing, (1998)). The packaging domain was replaced by a hexamer in the forward orientation of AVI, AII and AI, respectively.
20 The parent virus was nonviable (described Schmid, et al. (1997)), and lacking any functional packaging elements. Insertion of a hexamer of AVI, AII and AI into the 194/814 deletion background rescued virus viability,
25 albeit to different extents. A multimer of A repeat VI in place of the packaging domain resulted in a virus that exhibited a more than 100-fold reduction in growth in a single infection relative to wild-type virus. DNA packaging in a coinfection with wild-type virus was
30 nondetectable. A virus with 12 copies or more of AVI packaged at wild type efficiency. A hexamer of repeat I supported viral growth in a single infection and DNA packaging in a coinfection better than A repeat II, with
35 a reduction in growth of 4-fold versus 20-fold in the

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° single infection and in packaging efficiency of 2-fold versus 5-fold in the coinfection, respectively. These results suggest that there is a hierarchy of functional importance within the group of most efficient packaging
5 elements, with element VI as the weakest element followed by A repeat II and finally A repeat I as the functionally most dominant A repeat.

EXAMPLE 2

10 P complex purification scheme (Figure 5).
HeLa cell pellets were obtained from the National Cell Culture Center (Minneapolis, MN). All procedures were performed at 4°C. Nuclear extract was prepared by the
15 method of Dignam et al. (1983), dialyzed into buffer DB-100 (20 mM HEPES, pH 7.5, 100 mM NaCl, 5 mM MgCl₂, 20% glycerol, 0.1 mM EDTA, 0.5 mM PMSF, 0.25 mM benzamidine, 1.0 mM DTT), and the dialysate centrifuged at 25,000 x g for 20 minutes. Buffer DB is the same buffer but lacks
20 NaCl. Nuclear extract was applied to a heparin-agarose column (10 mg protein/1 ml heparin-agarose) equilibrated in DB-100, the column was washed with DB-100, and bound proteins were eluted with a linear NaCl gradient (0.1 M-0.6 M) in DB. Fractions containing P complex activity
25 were identified using a gel mobility shift assay with a DNA probe consisting of a dimer of A repeats V-VII (as per Fig. 3). The P complex peak eluted at 0.42 M NaCl. The NaCl concentration was diluted to 0.05 M using DB,
30 and the P complex pool was applied to a phosphocellulose P11 column (8 mg protein/1 ml P11) equilibrated in DB-0.05, the column was washed with DB-0.05, and bound proteins were eluted with a linear NaCl gradient (0.05 M-0.6 M) in DB. The peak of P complex activity eluted
35

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at 0.13 M NaCl. The P complex pool was diluted to 0.1 M NaCl using DB, and applied to an SP-Sepharose column (8 mg protein/1 ml SP-Sepharose) equilibrated in DB-100. The column was washed with DB-100, and bound proteins eluted with a linear NaCl gradient (0.1 M-0.6 M) in DB. The peak of P complex activity eluted at 0.20 M NaCl. The P complex pool was diluted to 0.1 M NaCl using DB and protease inhibitors aprotinin and leupeptin were added to 1 µg/ml to all buffers from this point on. The P complex pool was applied to a Q-Sepharose column (8 mg protein/1 ml Q-Sepharose) equilibrated in DB-100. The column was washed with DB-100, and bound proteins eluted with a linear NaCl gradient (0.1 M-0.6 M) in DB. The peak of P complex activity eluted at 0.28 M NaCl. The P complex pool was diluted to 0.1 M NaCl using DB, and NaPO₄ was added to 10 mM. The P complex pool was applied to a hydroxy-apatite column (5 mg/protein/1 ml hydroxy-apatite) equilibrated in DB-100+ 10 mM NaPO₄. The column was washed with DB-100 + NaPO₄, and bound proteins eluted with a linear NaPO₄ gradient (10 mM - 250 mM) in DB-100. P complex activity was pooled with a final purification of -1000-fold.

EXAMPLE 3

A cellular complex (P-complex) interacts with adenovirus packaging elements. Minimal packaging domains defined *in vivo* were used as probes for gel mobility shift assays for the detection of *trans*-acting packaging components. Since such components could be viral and/or cellular in origin, we initially carried out binding studies with both uninfected and Ad-infected 293 cell nuclear extracts. Infections were performed

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using either wild-type Ad dl309 or a temperature-sensitive virus, ts19, defective for virus assembly when grown at the restrictive temperature (Williams, et al. (1971)). Extracts from ts19-infected cells were tested in view of the fact that packaging factors may be encapsidated with wild-type adenovirus and consequently not present in nuclear extracts used for *in vitro* binding studies. At no point did we detect any difference between complex formation using nuclear extracts from infected or uninfected cells, and therefore, all experiments presented below were performed with extracts from uninfected cells.

A fragment containing a dimer of A repeats V-VII confers wild-type packaging abilities *in vivo* to a mutant virus which lacks the packaging domain (Schmid, et al. (1997)). Figure 3 shows the results from a gel mobility shift assay in which this fragment was used as a probe and incubated with uninfected 293 cell nuclear extract for the detection of interacting proteins. In lanes 1 and 24 (+), no specific competitor was added, whereas a 40- and 200-fold molar excess of competitor oligonucleotides were added to the binding reactions resolved in lanes 2 to 23. The specific competitor fragments are indicated above the autoradiography and represent different multimeric A repeats, either in the wild-type or mutated configuration (see Materials and Methods for names and sequences). A slow migrating complex, termed the P-complex (indicated by an arrow) was formed on the AV-VII probe (lanes 1 and 24), which disappeared upon self-competition (lanes 2 and 3), but not when the TTTG half-site of the packaging element

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consensus motif was mutated in A repeats V and VI of the competitor oligonucleotide (lanes 4 and 5).

In a similar fashion, the addition of fragments representing AVI (lanes 6 and 7), AV (lanes 10 and 11), AI (lanes 16 and 17) and AII (lanes 20 and 21) resulted in competition for P-complex formation, but not when the consensus TTTG half-sites were mutated (lanes 7, 8, 12, 13, 18, 19, 22 and 23). The efficiency of individual A repeats to compete for P-complex binding in a gel shift assay can be rated, with AV-VII and AI as the best competitors, followed by AII as an intermediate competitor and AVI as the weakest competitor. This correlates with the ability of the respective fragments to function individually as packaging domains *in vivo* (Fig. 2). Mutating the CG dinucleotide within the competitor oligonucleotide did not affect complex formation as exemplified by efficient competition observed with the AVCG competitor oligonucleotide (lanes 14 and 15) indicating that the CG consensus half site is not critically involved in P-complex binding. Other competitor oligonucleotides representing different A repeats with mutations in the CG dinucleotide were also tested, and identical results were obtained. P-complex formation was also observed using HeLa cell nuclear extract.

In summary, a cellular binding activity, termed P-complex, interacts specifically with various packaging elements in a gel mobility shift assay, in perfect correlation with data obtained *in vivo* with mutant viruses containing minimal packaging domains. Integrity of the AT-rich, but not the CG-rich, part of

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- ° the packaging consensus motif is critical for this interaction.

EXAMPLE 4

5 P complex interacts with viral core origin sequences. P complex binding activity was bound to bind to sequences derived from the left terminus of the adenovirus genome (Schmid and Hearing, 1998). Using gel mobility shift assays, the binding of P complex to A repeat sequences (AI hexamer probe or AV to VII dimer probe) was efficiently competed by an oligonucleotide containing left en ITR sequences from nucleotides 1 to 13, but not by an oligonucleotide containing ITR sequences from nucleotide 10 to 22. Similarly, P complex bound efficiently to a DNA probe containing ITR sequences from nucleotides 1 to 13, and this binding was efficiently competed by wild type A repeat oligonucleotide competitors, but not by A repeats with mutation in the TTTG consensus motif. The data show that P complex not only binds to packaging A repeats, but also to the very terminus of the adenovirus genome (nucleotides 1 to 13). As depicted in Fig. 4, the binding of P complex to the packaging domain and left terminus of the adenovirus genome followed by P complex protein-protein interaction may result in looping of the intervening DNA sequences. The competition experiments are consistent with one or two possibilities for P complex binding activity. First (Fig. 4 LEFT), P complex may contain one DNA binding activity that recognizes both packaging A repeats as well as the left terminus of the adenovirus genome (which is AT-rich but does not have a consensus A repeat sequence). Second (Fig. 4

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RIGHT), P complex may consist of two distinct but interacting activities whereby one DNA binding activity binds the consensus A repeat sequence and the second DNA binding activity binds to the AT-rich left terminus of the adenovirus genome.

EXAMPLE 5

COUP-TF interacts with adenovirus packaging elements. Database searches revealed that the AVI probe contains highly conserved dimeric consensus binding sites for a cellular transcription factor, chicken ovalbumin upstream promoter transcription factor (COUP-TF; Cooney et al. (1992)). COUP-TF binds to the consensus sequence 5'-GGTCA-3' when situated as a direct or inverted repeat, with a preferred spacing of 1 base pair, and represented as perfect or imperfect versions of the consensus binding site. These binding sites overlap A repeat VI (5'-GGACTTTGACC-3'; the COUP-TF inverted repeat is underlined, and AVI is in bold), only the upper strand is indicated with the COUP half sites underlined and AVI indicated in bold case. Other A repeats contain similar sequence motifs, albeit with less resemblance to the dimeric COUP consensus.

In view of the conserved COUP-TF binding motif contained within AVI, we asked whether the multimeric protein-DNA complexes formed on the AVI probe in particular, but also complexes formed on other A repeats, might contain COUP-TF (Schmid and Hearing, 1998). Heparin agarose fractions were subjected to Western blot analysis using a polyclonal COUP-TF antiserum. A band of approximately 45Kd molecular size was detected in fractions 24 to 31, which represents a

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low-molecular form of COUP-TF. The presence of COUP-TF protein in fractions 24 to 31 correlates with the presence of a packaging repeat binding activity which exhibits striking affinity for A repeat VI.

To test COUP-TF binding to A repeats I and VI directly, we performed gel mobility shift assays using *in vitro* transcribed and translated COUP-TFI with hexameric AVI and AI probes (Fig. 6). COUP-TFI strongly bound to the AVI probe (lanes 4 to 7), and weakly to the AI probe (lanes 13 to 16). Addition of polyclonal COUP-TF antiserum (lanes 9 and 18), but not preimmune serum (lanes 8 and 17), resulted in the formation of a supershift in each case. The formation of weak complexes on both probes by the addition of unprogrammed reticulocyte lysate alone (lanes 1 and 10) was observed. No supershifts, however, were formed upon the addition of either preimmune serum (lanes 2 and 11) or COUP-TF antiserum (lanes 3 and 12) suggesting that COUP-TF is not contained within these complexes. Probes representing AII and AV-VII bound COUP-TF with similar affinity to the AI probe. COUP-TF, when synthesized *in vitro*, displays sequence-specific binding affinity for all minimal packaging domains. COUP-TFI exhibits lowest binding affinity for AI and highest binding affinity for AVI, opposite to the ability of the respective elements to serve as minimal packaging domains *in vivo*.

Sequence-specific binding of COUP-TF to viral packaging elements provide another level of regulation of adenovirus packaging (Schmid and Hearing, 1998). COUP-TF binds to A repeats when synthesized *in vitro* (Fig. 6) or when expressed using baculovirus. Highest affinity was observed for A repeat VI multimers. Also,

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heparin agarose chromatography correlated the peak of binding activity interacting with A repeat VI multimers with peak levels of COUP protein. Further, gel mobility supershift experiments using minimal packaging domains as probes showed the presence of a COUP-related binding activity in uninfected nuclear 293 and Hela cell extracts.

EXAMPLE 6

To generate a "designer" adenovirus vector where COUP-TF specifically represses adenovirus packaging, a virus referred to as helper virus in Fig. 8 containing the USF-0 mutations in the MLP was generated. The salient features of the vector are: mutations of the COUP-TF binding site in the MLP (USF-0) so MLP activity is not repressed by COUP-TF overexpression *in vivo*, and 12 copies of A repeat VI in place of the normal adenovirus type 5 packaging domain (nt. 194-452). This new "designer" helper virus vector is termed USF-0 + AVI¹². A repeat VI is a high affinity COUP-TF binding site. USF-0 DNA or USF-0 + AVI¹² DNA was cotransfected with a COUP-TF high level expression vector (CMX-COUP-TF) or with a control vector (CMX) into human 293 cells. Two days later, production of infectious virus was assayed. The results (Fig. 9) showed that COUP-TF expression specifically repressed production of the "designer" virus USFO+AVI¹², with a minimal effect on the parent adenovirus USFO. The maximum level of expression of packaging of USFO+AVI¹² by COUP-TF expression was 400-fold.

Since our goal is to selectively repress adenovirus packaging using COUP-TF expression and

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0 binding to specific packaging sequences, elimination
COUP-TF repression of the adenovirus MLP was used to
demonstrate its effect on viral packaging. A viable
adenovirus mutant (termed USF-0; Reach et al. 1990)
5 which contains mutations in the USF binding site was
utilized. Binding studies showed that the USF-0
mutations disrupted the binding of COUP-TF to the MLP.
Importantly, COUP-TF was not able to repress the USF-0
virus when tested for infectious virus yield *in vivo* and
10 when MLP activity was analyzed *in vivo* in conjunction
with COUP-TF overexpression (See Figure 9(c)).
Importantly, COUP-TF expression had a minimal impact of
viral late gene expression indicating the specific
repression of packaging of USFO+AVI¹².
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EXAMPLE 7

Repression of adenovirus packaging by the lac repressor. Figure 10C depicts a "designer" adenovirus
20 vector whereby a binding site for the bacterial lac
repressor is situated adjacent to and overlapping
adenovirus packaging repeat AV. The lac repressor
binding site is a perfectly symmetric sequence that
25 binds lac repressor very tightly (Sadler et al. 1983).
Lac repressor is a bacterial protein not expressed in
eukaryotic cells. Eukaryotic cell, high level
expression vectors were generated in our laboratory that
express two forms of the lac repressor: 1) the wild type
30 lac repressor, 2) the X86 mutant lac repressor which
binds with 40-fold greater affinity to a lac site than
the wild type lac repressor. Both forms of lac
repressor carry epitope-tag (M45) at the amino-terminus
35 for detection of protein expression in eukaryotic cells

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by Western blot analysis using a monoclonal antibody against the epitope-tag (mAb M45; Obert et al. 1994). Fig. 11 shows a Western blot analysis of lac repressor expression in transfected 293 cells showing stable and high level expression of wild type and X86 lac repressors. Fig. 11 also shows a gel mobility shift assay using wild type and X86 Lac repressors expressed *in vivo* with a DNA probe containing the sequence shown in Fig. 10C. Stable DNA binding to the probe by both repressor forms is evident; specificity for Lac repressor is verified since: a) no binding is evident in cell extracts lacking Lac repressor, and b) the monoclonal antibody against Lac repressor alters the mobility (supershifts) the DNA-protein complex.

A recombinant adenovirus was generated that contains two copies of AV-VII + lac (Fig. 10C) in place of the adenovirus type 5 packaging domain (nt 194-814). The virus is viable and successfully propagated. AV-VII + lac viral DNA was cotransfected with the Lac repressor wild type high level expression vector (CMX + lac repressor) or with a control vector (CMX) into human 293 cells. Two days later, production of infectious virus was assayed. The results (Fig. 12) showed that lac repressor expression specifically repressed production of the "designer" virus AV-VII+lac. The maximum level of repression of packaging of AV-VII+lac by lac repressor expression was 20-fold.

EXAMPLE 8

The binding of P complex to A repeat sequences *in vitro* is dramatically reduced in the presence of ATP + MgCl₂. That is, the addition of 1 mM ATP + 2-10 mM

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° MgCl₂ to a standard DNA binding reaction with an A repeat DNA probe results in near total elimination of the P complex binding activity. This effect is not observed when a non-hydrolyzable analogue of ATP is used (gamma-S-ATP), thus ATP hydrolysis is involved in this process. P complex binds to AT-rich A repeat DNA sequences. An abundant cellular, nuclear protein that binds to such sequences is the TATA binding protein (TBP) which is a cellular transcription factor involved in transcription of cellular promoters. P complex binding is specifically competed using a known, high affinity TBP binding site (TATA box) which is supportive of the idea that P complex may contain TBP. P complex also binds to the adenovirus terminus to sequences 1-13. A panel of site-directed points mutations was made through this region to identify the binding site and it was found that all but one of the individual mutations did not reduce P complex binding, while combinations of multiple mutations reduced P complex binding 10-fold or greater. This type of binding pattern is consistent of a protein making interactions with the minor groove of the DNA, instead of the major groove of the DNA. It is known that TBP binds to the minor groove of DNA.

A protein complex containing TBP plus another protein termed TAF172 has been described (alternatively named TAF170; Timmers et al. 1992, Taggart et al. 1992). Both TBP and TAF172/170 are cloned (Hoffman et al., 1990, Kao et al. 1990, Knaap et al. 1997, Chicca et al. 1998). TAF172 has intrinsic ATP'ase activity and the TBP-TAF172 complex is displaced from DNA in the presence of ATP + MgCl₂, as found with P complex and A repeat binding (described above). A purification scheme, for P

- 45 -

° complex activity has been developed (Fig. 5). P complex
(identified by mobility shift assay with a DNA probe)
and TBP-TAF172 complex (identified by Western blot using
anti-TBP and anti-TAF172 antibodies) copurify through
5 each column used with a final P complex purification of
~1000-fold. Taken together, these results indicate that
P complex contains TBP-TAF172.

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CLAIMS

1. A method of regulating adenovirus packaging comprising the steps of:

a. obtaining an adenovirus vector containing a repressor binding site;

b. propagating said vector in the absence of said repressor; and

c. repressing packaging of said vector in the presence of repressor.

2. The method of claim 1 wherein the repressor is COUP-TF.

3. The method of claim 1 wherein the repressor is lac repressor.

4. The method according to claim 1 wherein the propagating step occurs in a first cell line and the repressing step occurs in a second cell line.

5. The method of claim 1 wherein the repressing step occurs in a cell line is coinfecting with a vector expressing the repressor.

6. An adenovirus vector comprising an adenovirus packaging sequence containing a plurality of COUP-TF binding sites.

7. An adenovirus vector comprising an adenovirus packaging sequence having at least two copies of 5'-TTTGN₈CG-3' and a plurality of COUP-TF binding sites.

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8. An adenovirus vector according to claims 6 or 7 further comprising a heterologous gene for expression in a host.

9. A method of treating patients comprising the step of:

administering an adenovirus vector that was prepared using the adenovirus vector of claim 8 wherein the heterologous gene expresses a therapeutically effective amount of a protein.

10. An adenovirus vector containing a packaging signal sequence consisting of at least two copies of 5'-TTTGN₈CG-3'.

11. An adenovirus vector according to claim 10 wherein a repressor binding site is embedded in the packaging signal sequence.

12. An adenovirus vector according to claim 10 wherein repressor binding sites flank the packaging signal sequence.

13. An adenovirus vector according to claim 10 wherein repressor binding sites alternate with the packaging signal sequence.

14. An adenovirus vector according to claim 10 having 3-12 packaging signal sequences.

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15. An adenovirus vector according to claim 14 wherein a repressor binding site is located between packaging signal sequences.

5

16. An adenovirus vector according to claim 11 or 15 wherein the repressor binding site is a *lac* repressor site.

10

17. An adenovirus vector according to claims 11 or 15 wherein the repressor binding site is a E2F binding site.

15

18. An adenovirus vector according to claim 10 further comprising a heterologous gene for expression in a host.

20

19. A method of treating patients comprising the steps of:

administering an adenovirus vector that was prepared using the adenovirus vector of claim 18 wherein the heterologous gene expresses a therapeutically effective amount of a protein.

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20. A composition comprising P-complex.

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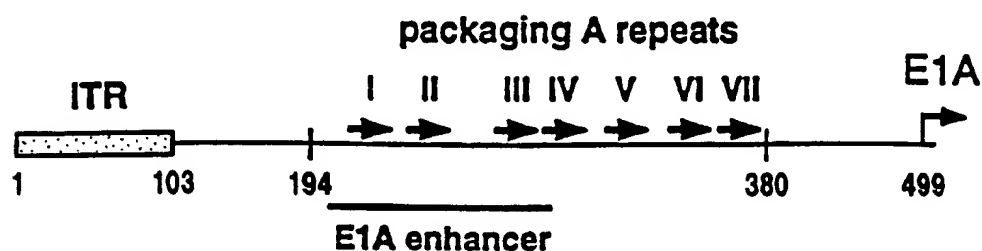


FIG. 1A

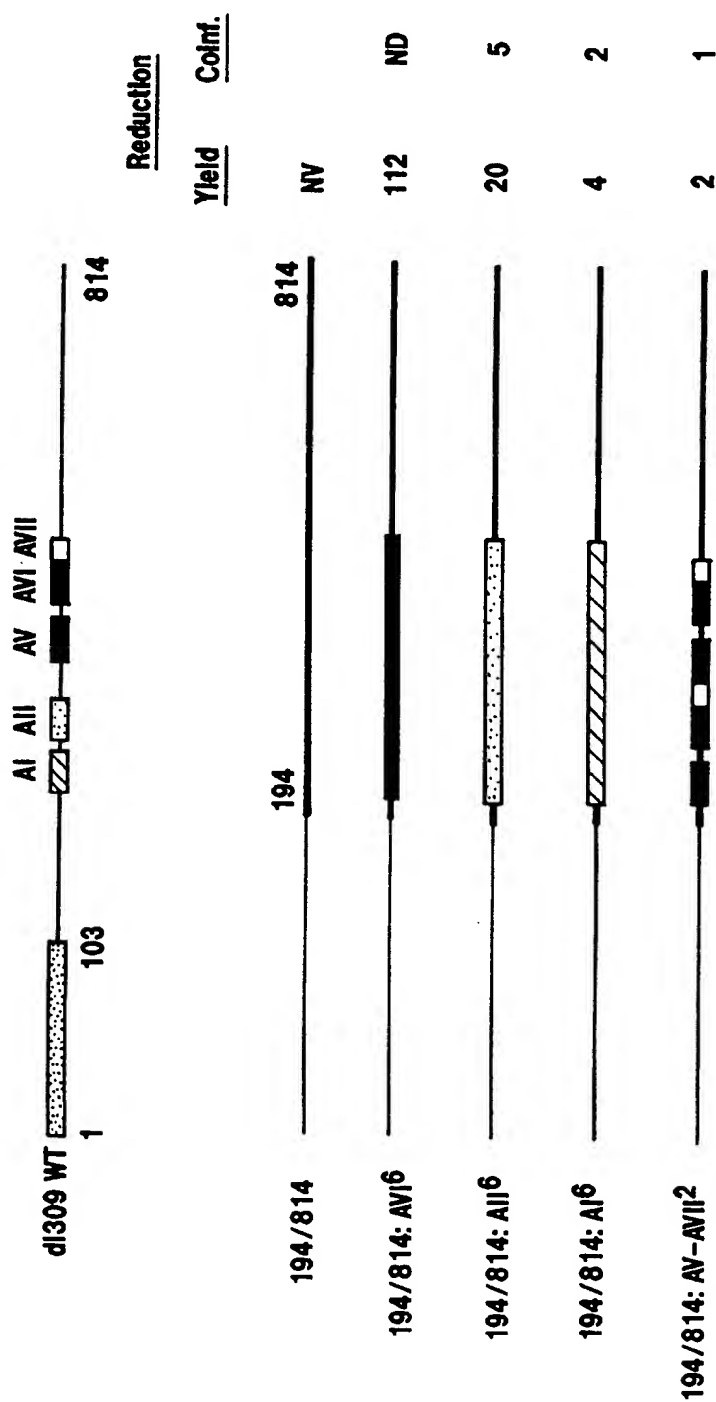
AI	TTTG	GGCGTAAC	CG
AII	TTTG	GCCATTTT	CG
AV	TTTG	TCTAGGGC	CG
AVI	TTTG	ACCGTTTA	CG

5'-TTTG N₈ CG-3'

FIG. 1B

	A V	A VI
Ad5	GCGCGTAATATTTGTCTAGGGCCGCGGGGACTTTGACCGTTTACGTGG	
Ad4	GGGAGGAGTATTTGCCGAGGGCCGAGTAGACTTTGACCGTTTACGTGG	
Ad12	GCGCGGAATATTTACCGAGGGCAGAGTGAACCTCTGAGCCTCTACGTGT	
Ad3	GGGTGGAGTATTTGCCGAGGGCCGAGTAGACTTTGACCGTTTACGTGG	
Ad9	GGGCGGAATATTTACCGAGGGCCGAG-AGACTTTGACCGATTACGTGG	

FIG. 1C



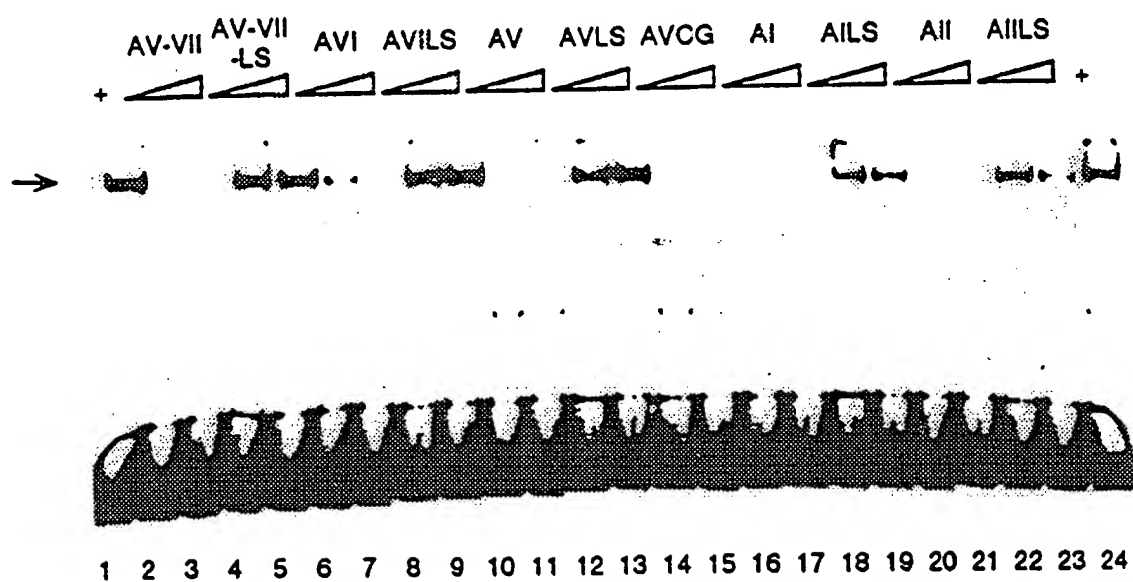


FIG. 3

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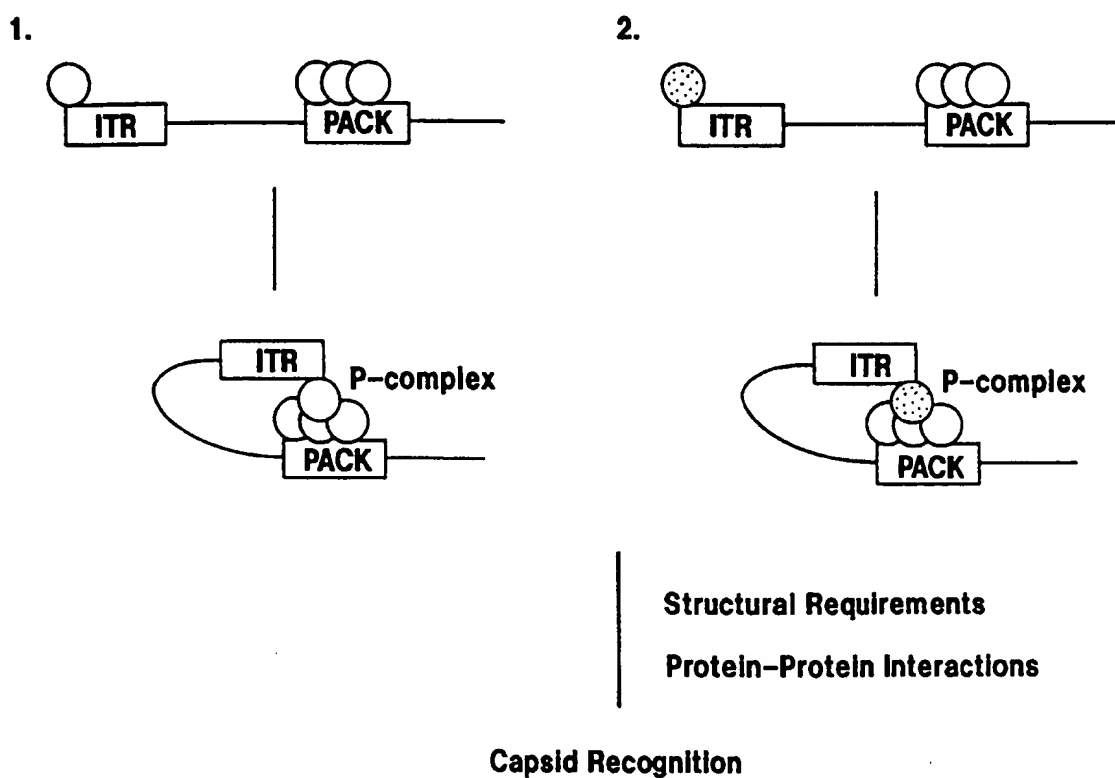


FIG. 4

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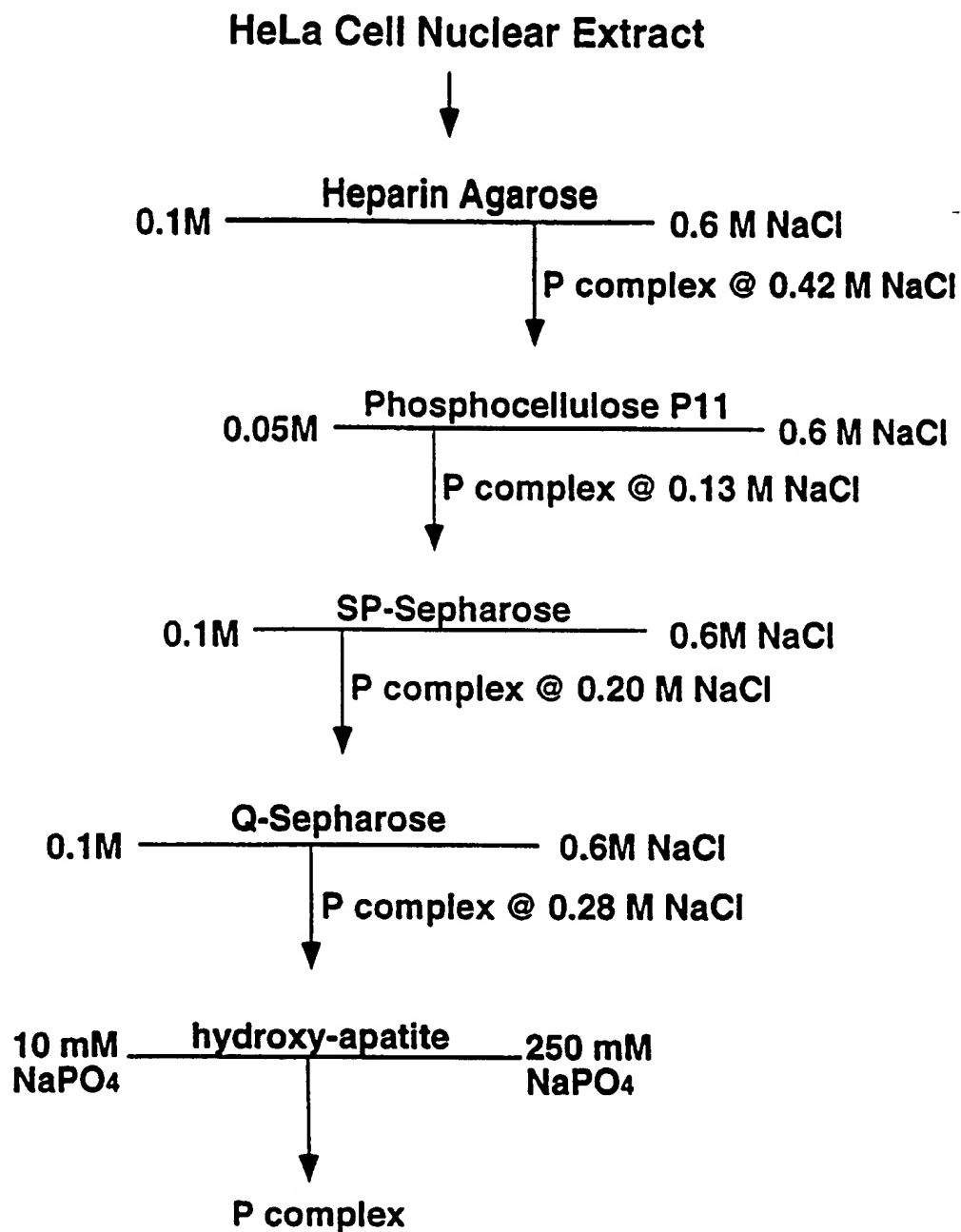


FIG. 5

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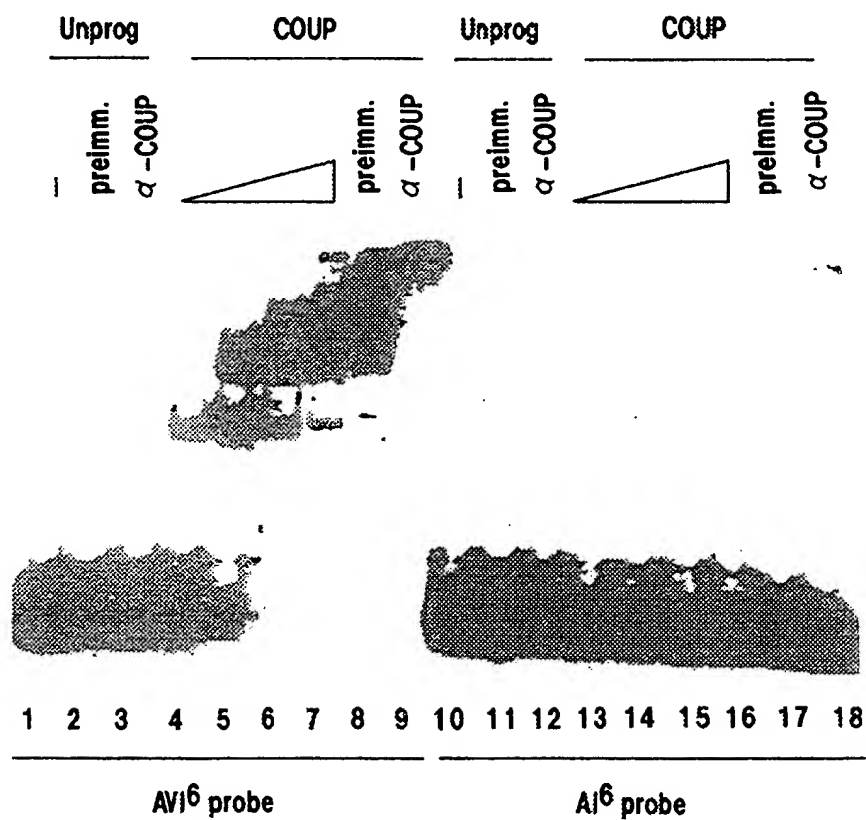


FIG. 6

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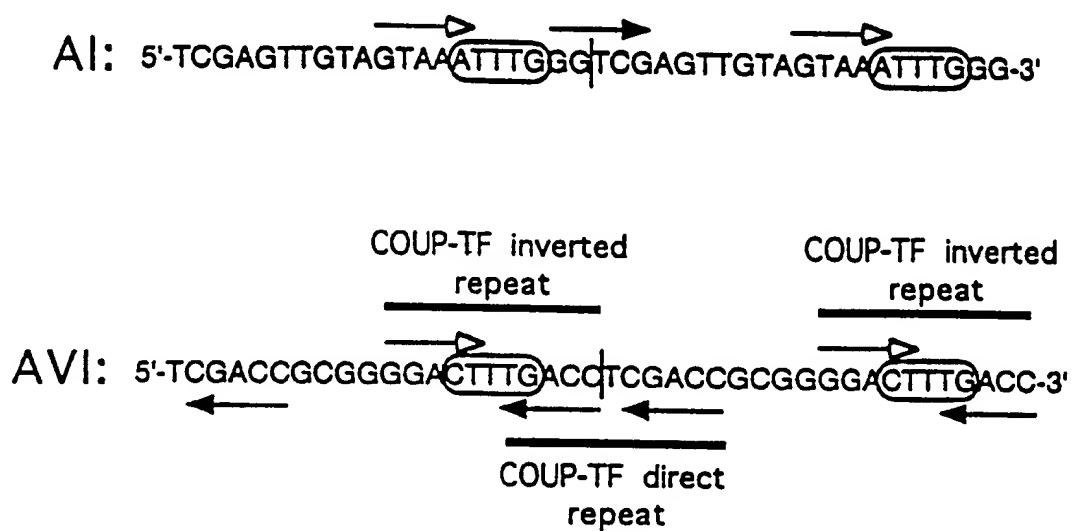


FIG. 7

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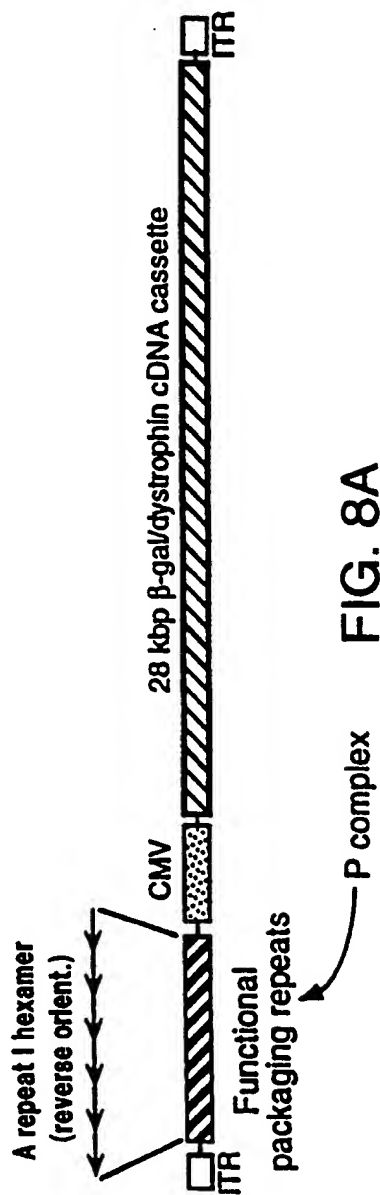


FIG. 8A

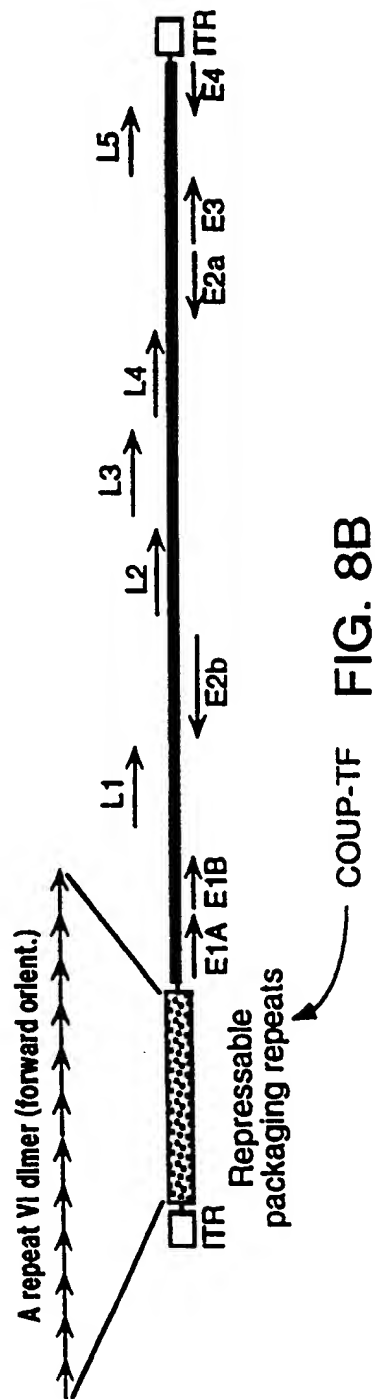


FIG. 8B

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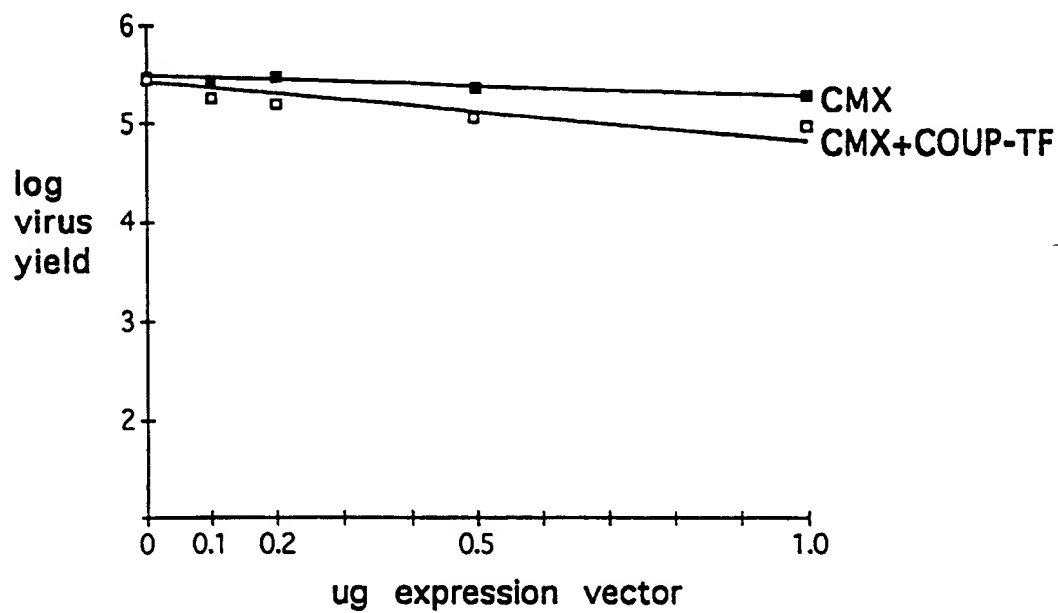


FIG. 9A

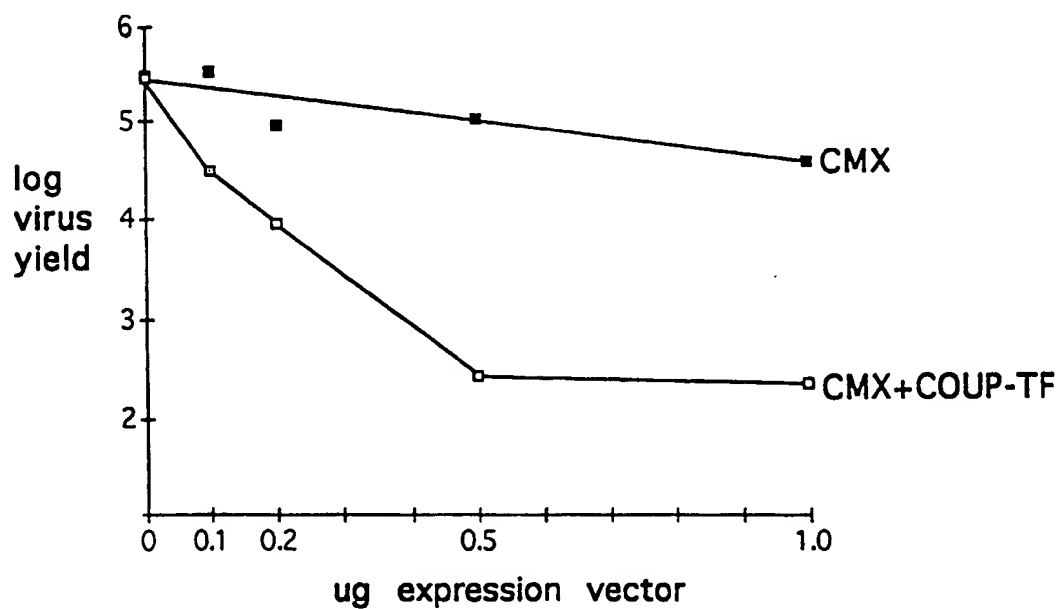


FIG. 9B

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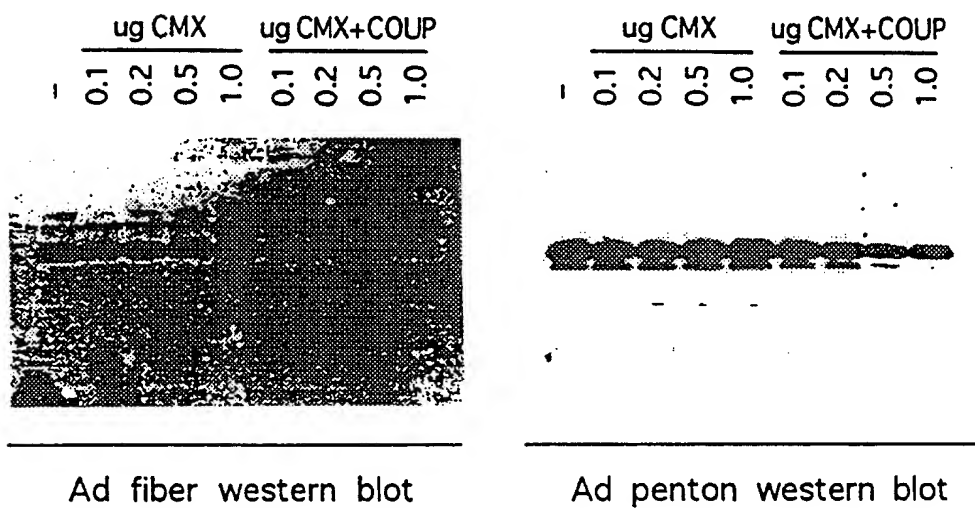
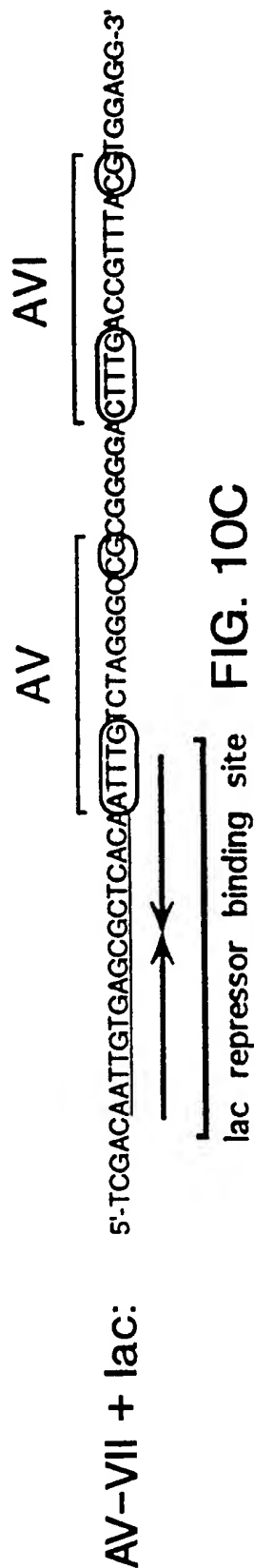
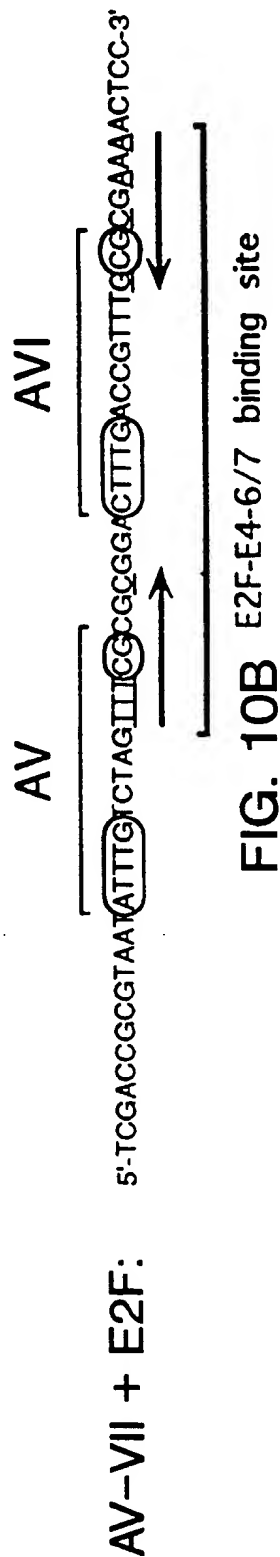
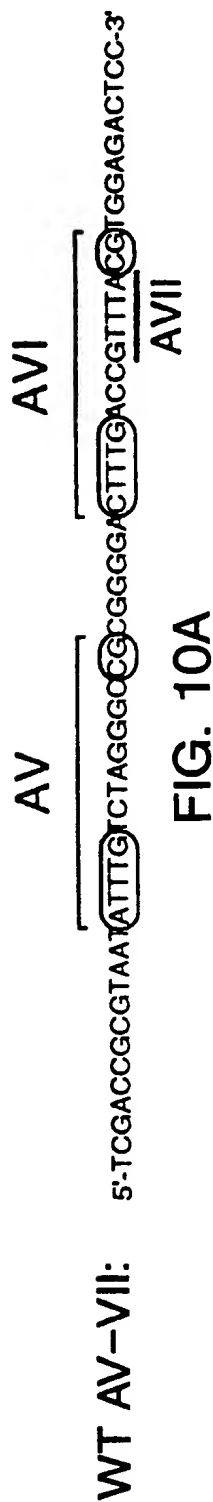


FIG. 9C

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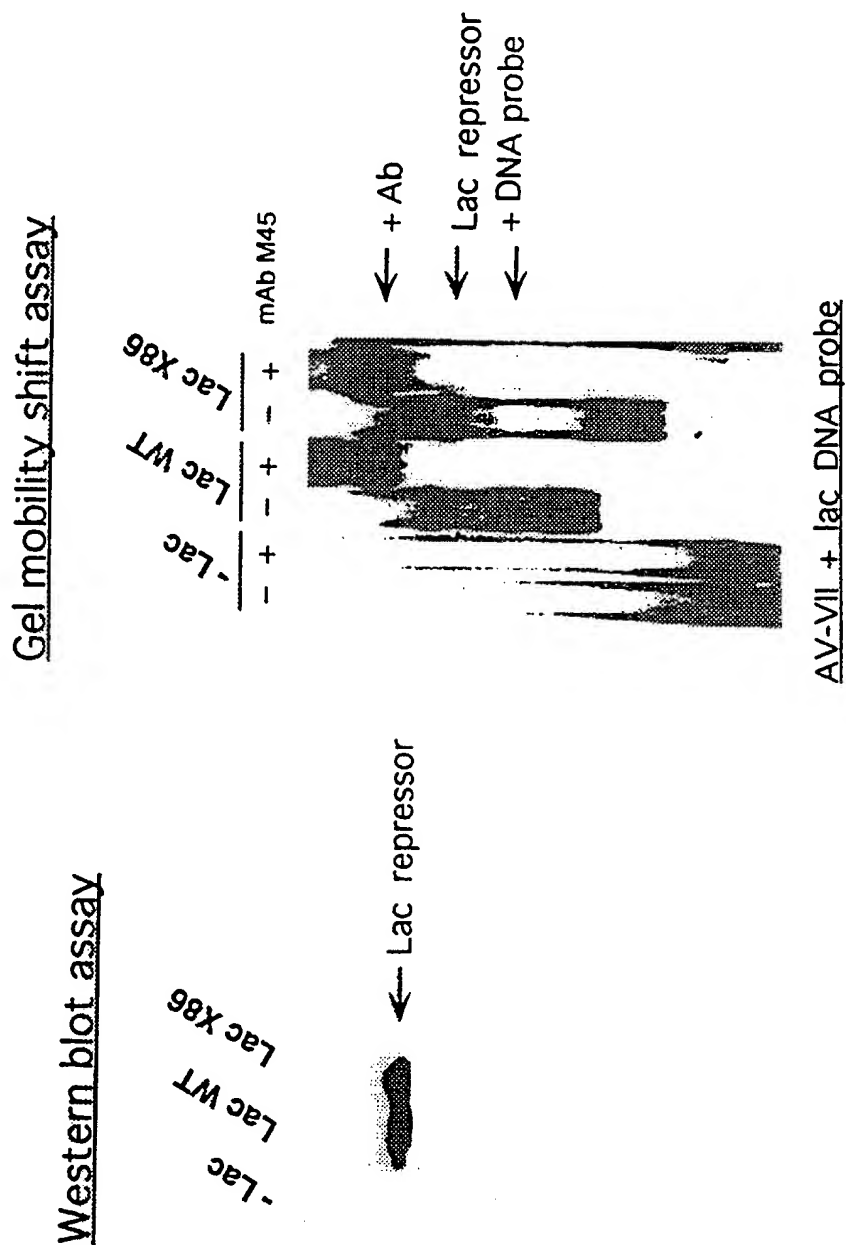


FIG. 11

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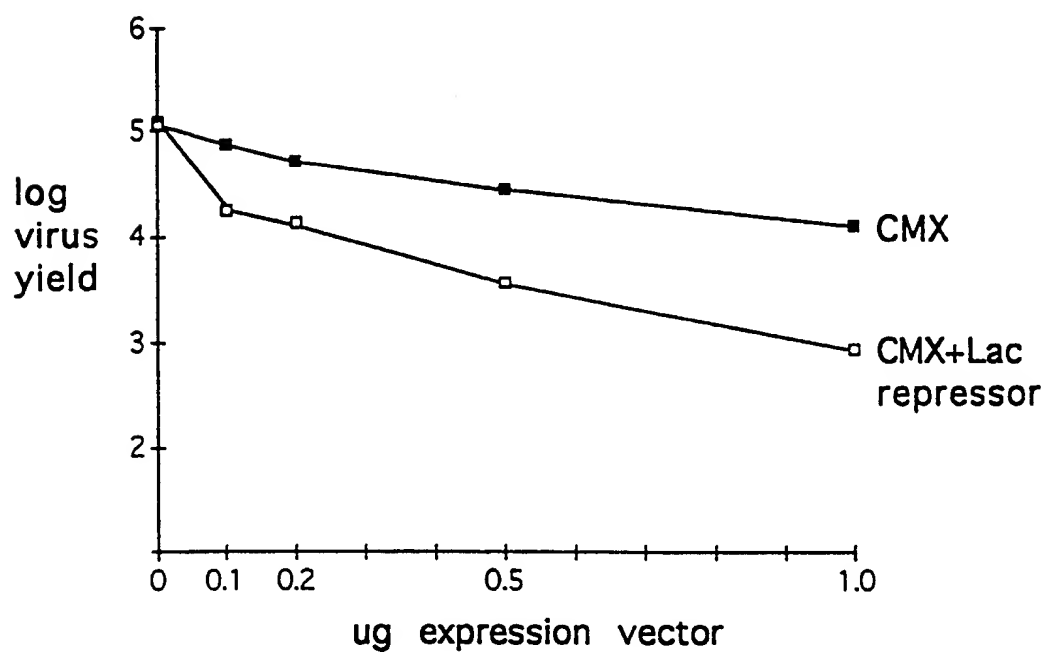


FIG. 12

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(21) International Application Number: PCT/US99/08294 (22) International Filing Date: 15 April 1999 (15.04.99) (30) Priority Data: 60/081,867 15 April 1998 (15.04.98) US 60/088,321 5 June 1998 (05.06.98) US (63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application US 60/088,321 (CIP) Filed on 5 June 1998 (05.06.98) (71) Applicant (for all designated States except US): THE RESEARCH FOUNDATION OF STATE UNIVERSITY OF NEW YORK [US/US]; P.O. Box 9, Albany, NY 12201 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): HEARING, Patrick [US/US]; 27 Thomas Hill Road, St. James, NY 11780 (US). SCHMID, Susanne, I. [DE/US]; Apartment 1, 2 Eliot Street, Somerville, MA 02143 (US). OSTAPCHUK, Philomena, H. [US/US]; 301 East Broadway, Port Jefferson,	(74) Agents: RICHTER, Kurt, E. et al.; Morgan & Finnegan, L.L.P., 345 Park Avenue, New York, NY 10154 (US). (81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> (88) Date of publication of the international search report: 27 January 2000 (27.01.00)	
(54) Title: SELECTIVE REGULATION OF ADENOVIRUS PRODUCTION		
(57) Abstract <p>The present invention relates to adenovirus vectors and their use in DNA delivery systems. The vectors have been designed to maximize their capacity to carry foreign DNA and to minimize the potential of producing replication competent virus. The vectors contain one or more copies of a minimum packaging sequence to direct virus packaging. Optionally, the vectors contain one or more repressor binding sites so that virion production can be selectively inhibited. Specific repression systems include COUP-TF and lac repressor. A cellular complex, called P complex is also disclosed. This complex functions positively in viral packaging and virus production.</p>		

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/08294

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/86

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SCHMID S.I., HEARING P.: "Selective encapsidation of adenovirus DNA" CURRENT TOPICS IN MICROBIOLOGY AND IMMUNOLOGY, vol. 199, no. 1, 1995, pages 67-80, XP002117827 the whole document	1
X	SCHMID S I AND HEARING P: "Bipartite structure and functional independence of adenovirus 5 packaging elements" JOURNAL OF VIROLOGY, vol. 71, no. 5, May 1997 (1997-05), pages 3375-3384 3384, XP002108134 ISSN: 0022-538X cited in the application the whole document especially figures 1-8 and discussion -/--	10,14, 18,19

☒ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search

2 November 1999

Date of mailing of the international search report

10/12/1999

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Panzica, G

INTERNATIONAL SEARCH REPORT

Inter national Application No

PCT/US 99/08294

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	O'CONNOR R J ET AL: "THE C-TERMINAL 70 AMINO ACIDS OF THE ADENOVIRUS E4-ORF6/7 PROTEIN ARE ESSENTIAL AND SUFFICIENT FOR E2F COMPLEX FORMATION" NUCLEIC ACIDS RESEARCH, vol. 19, no. 23, 11 December 1991 (1991-12-11), pages 6579-6586, XP000604601 ISSN: 0305-1048	15
X,P	--- SCHMID S.I., HEARING P.: "Cellular components interact with adenovirus type 5 minimal DNA packaging domains" JOURNAL OF VIROLOGY, vol. 72, no. 8, August 1998 (1998-08), pages 6339-6347, XP002117828 cited in the application the whole document	1,5, 7-15, 18-20
A,P	--- DULL T. ET AL.: "A third-generation lentivirus vector with a conditional packaging system" JOURNAL OF VIROLOGY, vol. 72, no. 11, October 1998 (1998-10), pages 8463-8471, XP002117829 BALTIMORE, US abstract	1
X	--- TAGGART A. ET AL.: "The TATA-binding protein and associated factors are components of Pol III transcription factor TFIIIB" CELL, vol. 71, no. 6, 1992, pages 1015-1028, XP002117830 abstract	20
X	--- D'HALLUIN J.C. ET AL.: "Effects of novobiocin on adenovirus DNA synthesis and encapsidation" NUCLEIC ACID RESEARCH, vol. 8, no. 7, 1980, pages 1625-1641, XP002117831 OXFORD, GB the whole document	1
A	--- HUANG M.M., HEARING P.: "The adenovirus early region 4 open reading frame 6/7 protein regulates the DNA binding activity of the cellular transcription factor, E2F, through a direct complex" GENES AND DEVELOPMENT, vol. 3, 1989, pages 1699-1710, XP002119862 cited in the application -----	

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 99/08294

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 19
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim 19
is directed to a method of treatment of the human/animal
body, the search has been carried out and based on the alleged
effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such
an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.